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CAMPHOR POISONING

ANATOMICAL AND PHARMACOLOGIC STUDY; REPORT OF A FATAL CASE; EXPERIMENTAL INVESTIGATION OF PROTECTIVE ACTION OF BARBITURATE *

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A fatal case of camphor poisoning has presented the stimulus for investigation of camphor intoxication by raising certain questions concerning the pharmacologic and biochemical mechanisms of the action of camphor. In this case there were anatomical features not reported previously in man. These specific questions and features are considered in the following presentation.

CAMPHOR POISONING IN MAN

In medical literature, there have been reports of at least 130 non-fatal cases of camphor poisoning, the majority occurring in the nineteenth century. Selected source references of non-fatal cases are listed in the bibliography.[1-21] Only 18 fatal cases of camphor poisoning have been reported,[22-34] and in only two of them were necropsy studies made. Despite the fact that symptoms referable to the central nervous system had dominated the clinical picture, cerebral anatomical changes have not been reported for man.

The two earlier necropsy studies concerned a 16-months-old male infant dying 7 hours after ingestion of a teaspoonful of camphorated oil,[26] and an adult woman dying 3 days after intraperitoneal injection of camphor.[33] In the former case, there was a profuse hemorrhagic eruption over the body surfaces, subperitoneal petechiae of the stomach and bowel, and subcapsular hemorrhages in the kidneys. In the latter case, changes were limited to congestion of the peritoneal surfaces and swelling of the proximal convoluted tubular epithelium in the kidneys. The mildness of this renal injury is of interest in view of the urinary retention, albuminuria, and anuria sometimes encountered in non-fatal

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cases. To these cases may be added the following example of fatal camphor poisoning in which severe changes occurred in the central nervous system.

Report of Case

A previously normal 19-months-old male infant swallowed an estimated 1 teaspoonful of camphorated oil (20 per cent camphor in cottonseed oil) from a liniment bottle. He vomited within a few minutes but remained otherwise asymptomatic until onset of salivation and rigidity 3 hours later. He was taken then to a local physician, who administered 50 mg. of demerol, and caffeine and sodium benzoate with little effect. The infant was hospitalized. His temperature was found to be 41.5° C.; pulse, 150; respirations, 28; blood pressure, 75/55 mm. of Hg. The white blood cell count was 40,000 per cmm., with 93 per cent segmented neutrophils and 7 per cent lymphocytes; the count was 21,600 per cmm. the following day. There was albumin in the urine at admission.

Shortly after hospitalization, the infant vomited 300 to 400 cc. of coffee-ground material which smelled of camphor. The subsequent clinical course was characterized by coma, repeated tonic convulsions, spasticity, and generalized hyperreflexia. Penicillin, fluid by hypodermoclysis, intermittent sedation by phenobarbital, nasal oxygen, and sponge baths were given. Two days prior to death the right pupil was fixed and dilated, and the blood pressure rose to 150/100. The child experienced recurring periods of apnea which increased in severity, requiring artificial respiration. Tracheotomy proved of little value, and the infant expired on April 16, 1951, 5 days following the ingestion of camphorated oil.

At necropsy 5 hours after death, there was atelectasis and edema of both lungs, with severe congestion of both lower lobes. The right side of the heart was dilated. A 1 cm. agonal or post-mortem perforation of the esophagus was found 2.5 cm. above the cardia of the stomach. There was focal mucosal congestion of the gastro-intestinal tract, but no odor of camphor. Central zonal congestion was observed grossly and microscopically in the liver, and the spleen and kidneys were congested. No other alterations were observed in the kidneys or bladder, either grossly or microscopically.

The brain was swollen and soft. It weighed 1,350 gm., or about 350 gm. more than normal. Microscopic study disclosed extensive degenerative changes selectively involving neurons and sparing glial and vascular structures. These changes were found diffusely distributed in the cerebral cortex and in the basal ganglia, and were most severe in Sommer's sector of the hippocampus, where virtually every pyramidal cell was necrotic. There was a small focus of ischemic necrosis in the medulla. The cerebellar Purkinje cells were unaffected.

The injured neurons exhibited all degrees of chromatolysis and nuclear pyknosis. Dead and dying neurons had a characteristic faded, ghost-like appearance in Nissl preparations (Figs. 1 and 2), and an intense eosinophilia with the hematoxylin and eosin stain (Fig. 3). The ground substance was loose and the perivascular and perineuronal spaces were enlarged, indicating edema. There was no associated glial

or vascular injury, no reactive hyperplasia, nor any inflammatory exudative reaction. The neuronal changes were similar to those found in severe anoxia.[35,36]

Since symptoms referable to the central nervous system have dominated the clinical picture in previous cases of camphor poisoning, the finding of cerebral anatomical changes in this case was not unexpected. It is probable that the lack of human necropsy data accounts for the lack of previous descriptions of these brain lesions in man. For instance, in the two previous necropsy studies, the brain of the adult was not examined and the short survival period of the infant would not allow the development of necrobiotic changes in neurons. In all likelihood, the child we report survived 4 days only because of the therapeutic effect of barbiturates. This 4-day period was sufficiently long to allow irreversible neuronal changes to become manifest anatomically.

Camphor Poisoning in Animals

Somatic changes reported in chronic experimental camphor poisoning in animals have included fatty alteration in the liver and kidneys.[37] Focal gastric ulcers, presumably secondary to contact points of concentrated solutions, have been observed following administration of camphor by stomach tube. Production of these ulcers has been connected theoretically with decreased glycuronic acid in the liver, due to conjugation with camphor and excretion by the kidneys. The decreased glycuronic acid was thought to be associated with hyposecretion of mucin by the stomach, thus exposing it to acid digestion.[38] Despite the report of a leukemoid reaction in a human patient who received camphor, no camphor effect on blood leukocytes in animals has been reported.[39]

Cerebral changes have been produced in animals by repeated toxic doses of camphor. Petechial hemorrhages, chromatolysis of nerve cells, focal zones of neuronal necrosis, and a reactive focal gliosis have been observed.[40] Other neuronal alterations, consisting of sclerosis (shrinkage, hyperchromatism, and clumping of the Nissl substance) also have been described.[41] However, these changes are of questionable nature, since they were found within a few hours following lethal doses of camphor. Furthermore, the latter changes appear identical to those described by Koenig and Koenig[42] as artifacts resulting from necropsy trauma, autolysis, and immersion fixation.

Experimental Camphor Poisoning of Animals

In an attempt to clarify the nature of the cerebral changes recorded in animals and to correlate these findings with those in our human

case, we studied the effect of camphor upon animals. Particular care was exercised to evaluate correctly the artifacts described by Koenig and Koenig,[42] which were regularly observed in our control animals as well as in rabbits or mice receiving camphor. These changes were found in animals dying in convulsions hours after administration of camphor as well as in those sacrificed several days after recovering from the convulsive episodes. The effects of single dose injections of camphor were studied in rabbits, and of single and multiple injections in mice.

Rabbits. The minimum lethal dose for rabbits is 2 gm. of camphor per kg. of rabbit.[43] Accordingly, 8 rabbits were given varying doses of camphor as camphorated oil (20 per cent camphor in cottonseed oil) by oral tube. Doses were 1/2, 2/3, 7/10, 8/10, 9/10, 1, 1½, and 2 MLD. In addition, 2 rabbits were given 25 per cent camphor in alcohol by gastric tube in doses of 8/10 and 9/10 MLD, and 10 control rabbits were given nothing. All rabbits receiving camphor had tonic and clonic convulsions within 5 to 40 minutes of ingestion. The tonic convulsions were characterized by rigidity and hyperextension of the forelegs, and the clonic by violent shaking motions of the entire body. Masticatory motions of the jaws, salivation, great hyperactivity, and violent jumping also were observed. Convulsions began later in the animals receiving the smaller doses. Those animals that died had convulsions intermittently until death; those that lived recovered from the convulsions within 4 hours and thereafter appeared normal. One rabbit receiving camphor in alcohol lived only 4 hours, but death was due probably to the aspiration of this material.

The brains were removed as soon as possible and were fixed by immersion in 10 per cent formalin within 30 minutes after death. Nissl preparations and celloidin and paraffin, hematoxylin and eosin preparations were studied. Other tissues were fixed in Helly's fluid.

Mice. An attempt was made to produce lesions of the central nervous system in mice by single injections of camphor and then by multiple injections. The LD/50 for mice was found to be approximately 30 mg. of camphor per 100 gm. (Mice nos. 13 to 32, Table I). All injections were given intraperitoneally. The results of these experiments are summarized in Table I. Mice nos. 1, 13 to 17, and 23 to 34 were necropsied and studied grossly and microscopically. Techniques for fixation and staining were similar to those used with rabbits.

All mice that had convulsions exhibited similar hyperactive behavior patterns, which consisted of marked clonic convulsions with some tonic elements, and running and jumping. One of the first signs to appear was rigidity of the tail, which would point straight up, such as is seen in the mouse in morphine poisoning.

Results

There were no significant lesions of the brain or spinal cord in the rabbits. The brains of all rabbits, including the controls, showed the artifacts described by Koenig and Koenig.[42] The esophageal and gastric mucosa of many of the rabbits was congested and showed small focal hemorrhages, but it was not certain whether these changes were due to camphor or trauma from the stomach tube. The kidneys of every rabbit were normal, as were the lungs, heart, liver, pancreas, and spleen.

In all of the mice listed in Table I, including both the ones that received single doses and those that received multiple doses, there were no significant lesions, other than those in the brain, attributable to camphor. All brains showed the pyknosis (hyperchromatism, sclerosis) of the neurons of the cortical layers described as a fixation artifact.[42] Mice nos. 13, 31, and 32 showed *bona fide* neuronal changes. The lesions consisted of necrosis of neurons in the brain stem, basal ganglia, medulla, hip-

pocampus, and cerebral cortex. These neuronal changes were virtually identical to those seen in the human case we report, as they were characterized by chromatolysis and nuclear pyknosis. Dead neurons exhibited an intense eosinophilia of the cytoplasm in the hematoxylin and eosin stains. In the Nissl preparations, necrotic cells were characterized by marked vacuolization of the cytoplasm and a loss of the cytoplasmic staining reaction.

The Mechanism of Camphor Action

The Pharmacologic Action of Camphor

The outstanding pharmacologic effect of camphor is stimulation of the central nervous system with excessive doses producing convulsions. The exact mechanism of the excitatory effect, the precise site of action,

Table I

The Effect of Camphor upon Rabbits and Mice

Animal	Camphor/kg.	No. of doses	Interval between doses	Length of life following first injection	Neuronal damage
	In cottonseed oil:				
Rabbit 1	1.0 gm.	1		5 days*	o
Rabbit 2	1.3 gm.	1		5 days*	o
Rabbit 3	1.4 gm.	1		5 days*	o
Rabbit 4	1.6 gm.	1		5 hours	o
Rabbit 5	1.8 gm.	1		40 hours	o
Rabbit 6	2.0 gm.	1		5½ hours	o
Rabbit 7	3.0 gm.	1		4 hours	o
Rabbit 8	4.0 gm.	1		45 minutes	o
	In 95% alcohol:				
Rabbit 9	1.6 gm.	1		4 hours	o
Rabbit 10	1.8 gm.	1		4 days*	o
Rabbits 11–20	o	o		o	o
	In 95% alcohol:				
Mice 1–5	1.8 gm.	1		5 minutes	o
	95% alcohol without camphor:				
Mice 6–10	0.6 cc.	1		5 minutes	o
	In cottonseed oil:				
Mouse 11	1.2 gm.	1		2 hrs., 15 minutes	o
Mouse 12	1.8 gm.	1		20 minutes	o
Mouse 13	0.30–0.40 gm.	3	24 hours	4 days*	++
Mice 14–17	0.30–0.40 gm.	3	24 hours	4 days*	o
Mice 18–22	0.30–0.40 gm.	1		1–3 hours	o
Mice 23–24	0.30–0.40 gm.	2	24 hours	25 hours	o
Mice 25–30	0.30–0.40 gm.	1		1–2 hours	o
Mouse 31	0.30–0.40 gm.	2	24 hours	36 hours*	++
Mouse 32	0.30–0.40 gm.	3	24 hours	56 hours*	+++
Mice 33–34	0.30–0.40 gm. +3 cc. Ringer's solution	2	24 hours	3 days*	o

* Animal sacrificed at specified time.

and the metabolic pathways involved are unknown. However, reactions are alike in man and other mammals in which the behavior patterns are known. The action of camphor upon frogs differs from that upon mammals by producing such rapid paralysis in frogs that convulsions do not occur.[44]

The action of camphor upon the circulation has been studied extensively.[1,45,46] Camphor, when given as a weak solution, causes vasodilatation of the coronary and peripheral arteries. Hence, convulsions, if caused by cerebral vasoconstriction, must be mediated through a mechanism other than that operating on peripheral arteries when camphor has been given as a weak solution.

Camphor has a carminative, as well as a rubefacient effect, and it induces several side reflexes, such as acceleration of the heart rate due to local irritative action on the oral mucous membrane. The drug is employed today as a liniment and insect repellent. It has been tried without real success as an abortifacient,[10,19,20,21,32] contraceptive,[14] cold remedy,[14] aphrodisiac,[21] anti-aphrodisiac, suppressor of lactation,[6] cardiac stimulant, and antiseptic.[1]

Camphor unites with glycuronic acid, perhaps in the liver, and is rendered inert. It may appear subsequently in the urine as camphoglycuronic acid.[45]

The fatal dose for man has been as low as 0.7 gm. in infants, but 15 gm. have been taken by adults without lethal effect. The oral MLD for rabbits is 2 gm. per kg.[43] and for guinea-pigs, 180 mg. per 100 gm.[43,47] Our tests indicated an LD/50 for mice of 30 to 40 mg. per 100 gm. when camphor was given intraperitoneally (Table I).

Cerebrovascular Mechanisms of Action of Camphor

Careful studies have been made of the possible vasoconstrictive actions of camphor. For example, study of data on cerebral blood flow obtained by inserting a thermorecorder into a cat's brain has revealed no general diminution in the flow during convulsions induced by camphor.[48] Studies of cerebral blood flow in animals given diodrast and similar convulsants have indicated early vasodilatation rather than constriction.[49] These facts provide experimental proof that vasospasm and anoxia are not requisite for the production of neuronal necrosis. Nevertheless, available experimental data are somewhat conflicting. Finesinger and Cobb[50] found, by direct observation of pial arteries, that homocamfin convulsions were preceded by slight constriction of the pial arteries, a drop in systemic arterial blood pressure, and a decrease in pressure of the cerebrospinal fluid. They found also that monobromated camphor convulsions were preceded by dilatation of the pial arteries, and, as a rule, an increase in pressure of the cerebrospinal fluid.

We have attempted to evaluate the relation of vasoconstriction to camphor action by anatomical study of the cerebral circulation before, during, and following camphor convulsions as compared to that in

normal animals. Two animals were killed by decapitation at each of these stages, and two control animals were sacrificed by decapitation. The benzidine method of Doherty, Suh, and Alexander[51] was used to demonstrate the state of the cortical blood supply. Our results gave no evidence of vasospasm, there being no demonstrable cerebral capillary anemia at any stage of camphor intoxication. When muscle spasm is eliminated by the use of curare or erythroidine, a severe depression of oxygen consumption by the brain still occurs,[52-54] thus indicating a probable mechanism of camphor action other than vasoconstriction. We conclude that vasospasm has little to do with the production of neuronal necrosis by camphor.

Biochemical Mechanisms of Action of Camphor

The biochemical mechanism by which camphor effects convulsions is unknown. Certain phenomena are known to be associated with convulsions, but as yet complete cycles of action of but few convulsants have been determined. With convulsions, there is an immediate increase in oxygen consumption.[49,52,55,56] Venous oxygen levels of the brain fall markedly during convulsions, independent of oxygen levels of arterial blood.[57] The lowering of arterial oxygen levels due to tonic spasm of respiratory muscles is of less importance than the local oxygen deficit due to excessive oxygen consumption.[52,55,58] Glucose consumption is increased and there are lower levels of phosphocreatine and of adenosine triphosphate during and following convulsions.[59-61] Inhibition of cholinesterase and consequent accumulation of acetylcholine are factors in the action of some convulsants,[52] and excessive formation of citrate,[62] disturbance of glutamic acid-glutamine equilibrium,[63] and effects due to liberation of ammonium ions are other alterations which have been related to convulsions.[63] Since oxygen and glucose are prime suppliers of expended energy, they are involved in the cycle of action of any convulsant. The biochemical agents utilized especially in this expenditure may be the high energy phosphate compounds such as phosphocreatine. Complete depletion of the intra-neuronal supply of these phosphate compounds may be the cause of neuronal death. These known alterations during convulsions do not explain how a convulsant initiates the chain of events that lead to excessive energy demands. Studies of the relation of camphor to oxygen or glucose consumption, or to the destruction of phosphate compounds, have not been performed *in vivo* or *in vitro*, but it may be assumed that camphor acts in a manner similar to that of most other convulsants.

Narcotics prevent the depletion of neuronal energy stores by the convulsant drugs. A knowledge of how this depletion is prevented

gives some idea of the biochemical cycles through which energy is transferred in convulsions, as well as some idea of how the depletion is prevented.[64,65] For instance, barbiturates inhibit oxidation,[66] probably in the cytochrome oxidase system at the cytochrome b–flavoprotein electron transfer level.[67-69] This oxidative interference is more pronounced in the gray matter than in the white matter, indicating a direct relation to the neuronal body rather than its axon. However, a similar action upon ganglionic oxidation affects transmission of the nerve impulse at the synapse.[70] The action of barbiturates cannot be localized completely to one process or to one oxidation level, since these drugs affect pyruvate, lactate, and glutamate oxidation as well as that of glucose.[66] Potassium ion concentration is known to affect the action of narcotics.[71] These many mechanisms and sites of action involved by narcotics and convulsants illustrate the difficulty of analyzing the mode of action of camphor on the basis of data now available.

Effect of Pentobarbital upon Animals Given Camphor

The following experiments on the action of barbiturates upon animals given camphor were undertaken to demonstrate the abolition of convulsions by barbiturates and to demonstrate the therapeutic value of barbiturates.

Ten mice were given camphor and pentobarbital intraperitoneally and a control series of 10 mice were given camphor only (Table II). The animals in the test series (mice nos. 35 to 44) became stuporous and then somnolent within 10 minutes, but again exhibited motor activity and complete recovery from the narcosis about 1½ hours after first administration of the drug. No animal had convulsions and, aside from the 1½ hour period of narcosis, they exhibited no abnormal behavior. In the control series, convulsions occurred in all mice.

The protective action of pentobarbital is demonstrated by the absence of fatality in mice given camphor and pentobarbital, as compared to seven deaths in 10 mice

TABLE II

Protective Action of Pentobarbital Against Camphor in Mice

Mice nos.	Camphor in oil	Pentobarbital	Narcosis	Convulsions*	Death*
	mg./100 gm.	*mg./100 gm.*			
35–44	30	7.2	1½ hours	0/10	0/10
45–54	30	0	0	10/10	7/10

* Number of mice having convulsions or dying over number of mice tested.

given camphor and saline solution. The 10 mice in the first series (nos. 35 to 44) were given similar doses of pentobarbital and camphor 24 hours after the first injection, and again 48 hours after the first injection. All 10 animals were killed 96 hours after the first injection, and were necropsied and their brains examined. No neuronal damage or other lesions were found. Similar anatomical studies were made on a control group of mice given only three successive daily doses of 1.8 mg. of nembutal. Again no lesions were found. It will be recalled from experiments reported in Table I

that repeated convulsions produced by multiple doses of camphor alone will produce neuronal necrosis in about 30 per cent of mice given two or more doses of camphor.

Mice nos. 1 to 10 demonstrated the lethal effect when 95 per cent alcohol was used as a solvent for camphor.

These experiments indicate that pentobarbital not only prevents convulsions but also protects the mouse from detectable tissue damage. The possibility that barbiturates at a therapeutic concentration might add by an anoxemic effect to the damage produced by camphor is considered unlikely in view of the protective action repeatedly demonstrated.

Summary and Conclusions

Knowledge of the pathologic anatomy of camphor poisoning is augmented by the report with necropsy findings of an additional human case. Of special significance is the observation of neuronal necrosis, a heretofore unreported finding in human camphor poisoning. Similar neuronal destruction was produced experimentally by multiple dose administration of camphor to mice.

The pharmacologic and biochemical actions of camphor are reviewed. The action of camphor was studied in relation to barbiturate antagonism of camphor. A possible conclusion which may be drawn from this study is that the site of action of camphor is intraneuronal and upon the oxidation cycle at a phase above the flavoprotein-cytochrome b level of the cytochrome oxidase system. Camphor probably acts in a manner similar to other convulsants, causing rapid oxidation and depletion of high energy phosphorus compounds, but the exact manner in which this high energy chain reaction is initiated is unknown.

Experiments were performed which demonstrate clearly the inhibition of camphor convulsions in mice by barbiturates. This is the *in vivo* counterpart to *in vitro* studies that demonstrate barbiturate inhibition of oxidation on brain substrates. Anatomical studies of mice given camphor and barbiturates demonstrate that with prevention of convulsions by barbiturates there is also protection against neuronal damage. Since barbiturates similarly suppressed convulsions in the fatal human case we report, we conclude that use of barbiturates in patients with camphor poisoning is therapeutically sound.

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LEGENDS FOR FIGURES

FIG. 1. Hippocampus, human case. Rarefaction and pallor of Sommer's sector of hippocampus resulting from neuronal necrosis. Nissl stain. × 11.

FIG. 2. Hippocampus, human case, showing selective neuronal necrosis of pyramidal layer. Arrow indicates two pale, ghost-like bodies of necrotic neurons, several of which are included in this field. Nissl stain. × 380.

FIG. 3. Central gray matter of hippocampus, human case, showing selective neuronal necrosis. Nuclear pyknosis and eosinophilic homogenization of the cell body are exhibited by all neurons but one in this field. Hematoxylin and eosin stain. × 700.

FIG. 4. Hippocampus, mouse 9, showing selective neuronal necrosis in dentate gyrus. The zone of homogeneous paleness of necrotic neurons contrasts sharply with the basophilia of the sector in which interstitial edema is the only alteration. Nissl stain. × 210.

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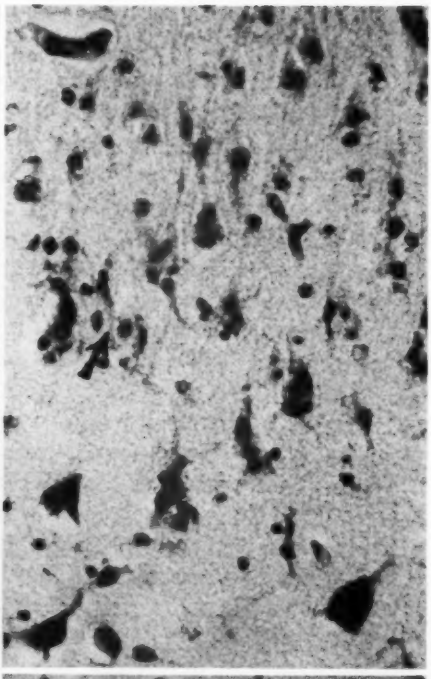

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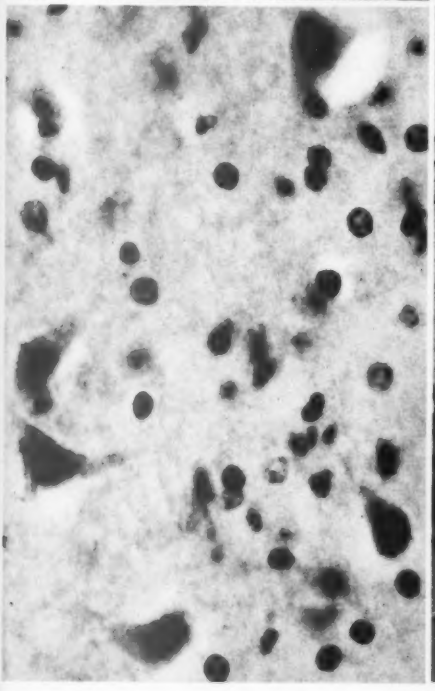

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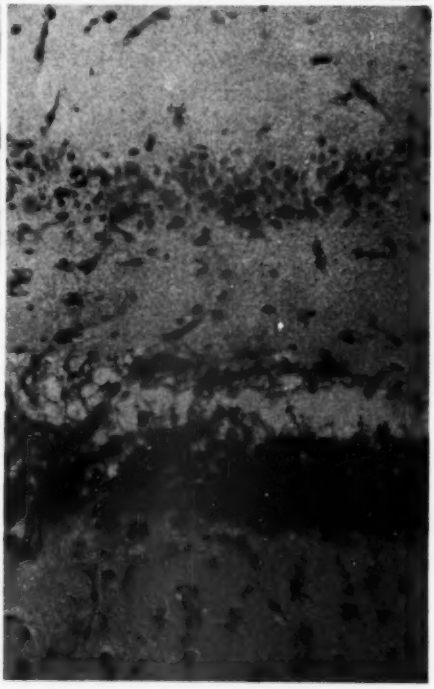

4

STELLATE INCLUSION BODIES IN PLASMA CELL MYELOMA AND IN GAUCHER'S DISEASE *

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Spiny intracytoplasmic bodies, generally termed "stellate inclusions" or "asteroid bodies," are observed rather commonly within reactive, multinucleated giant cells associated with granulomatous inflammation. Conditions in which they have been reported to occur, listed recently by Cunningham[1] and by Jaques,[2] include tuberculosis, blastomycosis, sarcoidosis, cryptococcosis, histoplasmosis, leprosy, talc granuloma, granulomatous mastitis, paraffinoma, adenomyoma of the uterus, granulomatous reactions in lymph nodes draining carcinoma, and foreign body reactions about dermoid cysts, suture material, and crystals of sodium biurate.

Previously summarized staining, chemical and physical properties of asteroid bodies after subjection to fixatives are as follows: (1) insoluble in water; (2) insoluble in fat solvents, including alcohol, ether, chloroform, benzene, xylene, acetone, gasoline, pyridine and a 1:1 mixture of xylene and glacial acetic acid; (3) insoluble in a 10 per cent solution of sodium hydroxide and in 10 per cent hydrochloric or formic acids; (4) isotropic in polarized light; (5) non-melting point to 350° C., but destroyed by heat between 350° and 650° C.; (6) staining pink or red with eosin, blue or purple with phosphotungstic acid-hematoxylin after fixation in Zenker's solution, positively with the Lorrain Smith-Dietrich method, central "core" positive and spines negative with Fischler's stain for fatty acids, negative to all other stains for lipids, including Sudan III, Sudan black, scarlet red, Nile blue sulfate, osmic acid, and the plasmal reaction; (7) negative to methods for carbohydrate, including use of the periodic acid-Schiff reagent; (8) negative for calcium and ferric iron; (9) negative for collagen and reticulin; (10) not silver-reducing, not acid-fast, and negative to the Feulgen reaction.[1-3]

Engle[4] has called attention to the frequent occurrence of stellate bodies in non-caseous granulomatous lesions associated with disorders of lipid metabolism, such as obesity, hypothyroidism, and diabetes mellitus. Jaques[2] has studied the relationship of these bodies to lipids and has proposed that they are composed of phospholipoprotein, although certain inconsistencies in their chemical reactions were acknowledged.

* Received for publication, January 13, 1954.

The purpose of this report is to record the observation of stellate inclusions within neoplastic multinucleated cells in an unusual case of plasma cell myeloma and in the giant "Gaucher's cells" in 2 cases of Gaucher's disease. In all 3 cases, these stellate inclusions have been morphologically identical with those commonly found in granulomatous inflammation. It is considered that these observations are of some value because these asteroid bodies have not, to my knowledge, been reported previously in these conditions; new lines of approach to the problem of the nature of these bodies are, therefore, opened. Some histochemical properties of stellate inclusions not previously described will be reported, and a concept of their morphogenesis will be submitted that does not appear currently to be under serious consideration.

Report of Cases

Case 1

A 68-year-old man of English descent entered the Mayo Clinic complaining of pain in the right forearm for about 6 weeks. His home physician had made a diagnosis of multiple myeloma, chiefly on the basis of findings in roentgenograms of the arms and skull.

Examination revealed a blood pressure of 180/80 mm. of Hg. A small tender mass was noted in the right forearm; this mass appeared to originate from the ulna. A lymph node in the left supraclavicular space was enlarged to about 2.5 cm. No other significant findings were present.

Roentgenograms of the thorax showed a mass in the left pulmonary hilus and a round density in the periphery of the upper portion of the left lung that appeared to be arising from the third rib. A destructive lesion interpreted as due to a malignant neoplasm was present in the vertebral end of the right fifth rib. The skull showed multiple zones of bony destruction throughout the calvarium, with similar but less extensive involvement of the mandible and facial bones. These changes were interpreted as characteristic of multiple myeloma, as were multiple, discrete, well circumscribed, destructive lesions found in the bones of both forearms.

Laboratory studies showed albuminuria graded 2, on the basis of 1 through 4. Bence Jones protein was identified. Hemoglobin measured 11.7 gm. per 100 cc. of blood; erythrocytes numbered 3,810,000 per cmm. of blood, and leukocytes numbered 5,700. Smears showed a moderate number of rouleaux and immaturity of granulocytes with an occasional myelocyte but no other significant change. The erythrocytic sedimentation rate was 44 mm. during the first hour (Westergren method). The total serum proteins measured 7.26 gm. per 100 cc.

Study of sternal marrow obtained by aspiration showed increased numbers of plasma cells, many of which were atypical and contained pink-staining granules in their cytoplasm. Sections of particles of marrow showed solid aggregates of plasma cells with rather voluminous pink-staining cytoplasm. These findings were considered diagnostic of multiple myeloma. Biopsy of the left supraclavicular lymph node confirmed the diagnosis of plasma cell myeloma. The patient was treated with urethane.

Case 2

A 36-year-old Jewish man, a physician, remarked in the course of an annual physical examination that he had noted a tendency to bruise easily for years. Examination revealed an area of ecchymosis, 5 cm. in diameter, over the lower part of the

abdomen; the tip of the spleen was palpable. Roentgenographic examination of the thorax, pelvis, femora, and spinal column disclosed no abnormalities.

Routine studies of urine and blood revealed no significant abnormalities except a reduction in platelets to 32,000 per cmm. of blood. Values for prothrombin time (Quick method) and serum bilirubin were within normal limits. A test of hepatic function showed no abnormal retention of sulfobromophthalein. The sternal marrow contained large numbers of typical Gaucher's cells, including many multinucleated forms. The megakaryocytes did not appear to be decreased.

Splenectomy was performed. The spleen weighed 665 gm. No capsular changes were noted. The cut surface was somewhat paler than normal, with multiple, darker, bulging areas up to 4 cm. in diameter. The microscopic picture in these zones revealed greatly dilated sinusoids virtually filled with mononucleated and multinucleated Gaucher's cells that showed the characteristic eosinophilic cytoplasm with delicate linear striations. Other regions in the spleen likewise contained Gaucher's cells in the sinusoids but did not show destruction of the sinusoidal architecture. An occasional megakaryocyte was present.

The blood platelets increased to a maximum of 180,000 per cmm. 4 days after operation; 8 months later they numbered 91,000. The patient remained asymptomatic and continued his practice.

Case 3

A 28-year-old Jewish man, a laboratory technician, entered the clinic stating that his spleen and liver were enlarged. He thought he had the same disease that his brother had had; the latter had undergone splenectomy previously at the clinic, at which time a histologic diagnosis of Gaucher's disease was made.

Examination revealed palpable lymph nodes in the left axilla, enlargement of the liver to 2 fingerbreadths below the xiphoid in the midline, and enlargement of the spleen, the edge being palpable slightly below the umbilicus and nearly in the midline. Roentgenograms of the skull showed no positive findings, but both femurs were considered to show rarefactive changes compatible with Gaucher's disease.

Laboratory studies showed albuminuria graded 1. Hemoglobin measured 11.4 gm. Erythrocytes numbered 3,800,000 and leukocytes 5,700; no significant changes were noted in the differential count. Blood platelets numbered 33,000. The erythrocytic sedimentation rate was 16 mm.

The sternal marrow contained large numbers of typical Gaucher's cells in smears, and nests of Gaucher's cells in sections.

Splenectomy was performed. The spleen weighed 1,900 gm. Except for its larger size, the gross and microscopic findings were identical with those described in case 2. Six days after operation, the platelets had increased to 328,000.

HISTOPATHOLOGIC OBSERVATIONS IN PLASMA CELL MYELOMA (CASE 1)

Both histologic and cytologic components of the lymph node were completely replaced by neoplastic cells. The picture was remarkable for the fact that, while the type of neoplastic cell was clearly a variant

of the plasma cell, it also displayed features of the histiocyte and, in numerous areas throughout the sections, progression of maturational changes occurred, ranging from immature "histiocytoid plasma cells" with relatively small volumes of cytoplasm to mature cells with massive, acidophilic, granular cytoplasm (Figs. 1 and 2). Cells of the latter type were reminiscent of the "storage" cells seen in Gaucher's disease. Giant multinucleated cells manifestly derived by conglomeration of numbers of these cells were numerous. The nuclei in such giant cells had no special arrangement within the syncytial mass; they numbered anywhere from two to more than 100 within that portion of the cell included in a slice 8 μ in thickness (Fig. 3). Nuclear and cytoplasmic fragmentation in some of these largest forms indicated these cells were dying or dead. Mitotic figures were found only in the mononucleated forms.

In general, only the smaller, less mature cells (Fig. 4) showed definite characteristics of plasma cells (peripheral clumping of nuclear chromatin, eccentricity of nuclei and basophilic cytoplasm with a Hof in cells stained by Dominici's technique[5]); the nuclei of the larger, more mature cells, including multinucleated ones, lacked many characteristics of plasma cells and resembled to a considerable degree the nuclei of epithelioid and giant cells as commonly seen in granulomas. This fact raised the question as to whether the multinucleated cells were actually derivatives of the neoplastic plasma cells or were simply reactive giant cells. It is not uncommon to observe foreign body giant cells in association with deposits of amyloid. The answer to this question was sought by means of careful examination of numerous sections from multiple blocks including all of the tissue removed. A number of staining and histochemical procedures were used, including Harris' hematoxylin and eosin,[5] toluidine blue,[6] acid fuchsin and basic fuchsin (each in a 0.5 per cent solution in 50 per cent alcohol), Dominici's stain,[5] van Gieson's stain for connective tissue,[5] Gömöri's stain for reticulin,[5] crystal violet,[5] Regaud's iron alum-hematoxylin,* the Hotchkiss[7] periodic acid-Schiff reaction with and without pretreatment with saliva, the Feulgen reaction,[5] the xanthoproteic reaction,[8] Millon's reaction,[9] and the arginine reaction (the Thomas modification of

* The actual technique used was a modification of that routinely employed in staining fixed smears in the Parasitology Laboratory of the Mayo Clinic. The steps were as follows: (1) deparaffinize sections and rinse in distilled water; (2) mordant for 30 to 45 minutes in a 4 per cent solution of ferric ammonium sulfate; (3) wash in running distilled water for 3 to 5 minutes; (4) stain for 45 minutes in Regaud's hematoxylin,[5] and (5) decolorize to the extent desired in a 2 per cent solution of ferric ammonium sulfate. Rinse in distilled water, wash in running water for 15 to 30 minutes, dehydrate, clear, and mount in Canada balsam.

Serra's method[10-12]). Observations with the aid of these methods left little reasonable doubt that the multinucleated cells, in which stellate inclusions were found, represented derivatives of neoplastic plasma cells and were not reactive, non-neoplastic giant cells.

Cytoplasm. Use of crystal violet and toluidine blue revealed no metachromatic intracellular or extracellular substances that could be interpreted as amyloid or mucinous material. These dyes showed progressively decreasing uptake in the cytoplasm as the cells became larger and multinucleated.

The presence of intracytoplasmic granules was demonstrable with all dyes and reactions used. These granules ranged in size from the limits of visual resolution up to 3 μ in diameter; they were rod-shaped or polygonal as a rule, but sometimes were nearly spherical. They stained deeply with eosin, acid fuchsin, and basic fuchsin. They stained extremely faintly with toluidine blue and crystal violet. They were colored a deep yellow with van Gieson's stain. Gömöri's reticulin method and Regaud's iron alum-hematoxylin both rendered the granules black; in the latter method, however, the dye could be removed by prolonged (15 minutes or more) destaining with the mordant. The Feulgen reaction was negative when applied to the granules. With the periodic acid-Schiff reaction, the granules became moderately pink; this reaction was not prevented by pretreatment with fresh saliva at 37° C. for 30 minutes. The granules became bright yellow when a drop of concentrated nitric acid was applied to a section (xanthoproteic reaction), were pale pink with the arginine method, and pale pink-orange with the Millon reagent. Cells of the plasma cell series present in Wright-stained smears of sternal marrow also displayed these granules, which stained pink.

These granules were demonstrated most clearly in sections stained by Dominici's method,[5] for here they stained deep pink to orange and were sharply contrasted against a background of blue in the less mature cells. With this stain, it was particularly evident that the granules were present even in the younger plasma cells, although they were less numerous and smaller in these cells than in more mature ones. As the characteristic basophilia of the aging plasma cells became less prominent, the acidophilic granules became more numerous, until in the largest single-nucleated cells they occupied practically all of the voluminous cytoplasm. By all indications, the multinucleated cells were formed as a result of fusion of such cells, for the cytoplasm of the multinucleated cells of all sizes was likewise packed with identical granules.

Nuclei. A progression of nuclear changes paralleled the progression of cytoplasmic changes. In sections stained with hematoxylin and eosin or by Dominici's method, the youngest forms of plasma cells displayed nuclei with relatively numerous coarse particles of chromatin distributed throughout the cytoplasm; a definite tendency toward the appearance of spokes in a wheel was noted in many of the cells (Fig. 4). The nuclear membrane in these cells was moderately delicate and regular. Usually a single, small, centrally located nucleolus with a slightly eosinophilic tint was present. Nucleoli were colorless in Feulgen preparations.

Concomitant with expansion of the cytoplasm and accumulation of acidophilic granules in the successively maturing stages, nuclei sometimes underwent enlargement, with the particles of chromatin becoming distributed more markedly toward the nuclear membrane (Fig. 5). At the same time, this membrane itself became thinner, as if stretched out, and the nucleolus larger (Fig. 6). In some of the cells, particularly in those with multiple nuclei, this process of nuclear expansion progressed until the nucleus reached more than twice the diameter of those of smaller and less mature cells (Fig. 6). Nuclei of this type contained, as a rule, proportionately large nucleoli that usually were located centrally but sometimes were adjacent to the nuclear membrane. Such nuclei furthermore showed much less prominence of chromatin in the central portions and had the appearance of being filled with a non-staining substance that compressed the chromatin into thin partition-like strands. This change was particularly evident in sections stained by Feulgen's method, which showed a pronounced "thinning out" of Feulgen-positive material.

The swollen type of nucleus was not invariably present in all of the more mature cells. On the contrary, nearly an equal proportion of mature cells, either mononucleated or multinucleated, contained small, pyknotic nuclei, although the cytoplasmic characteristics were the same. It appeared that, in the process of aging, either pyknotic or "hydropic" alteration might occur. In the large, multinucleated cells, both pyknotic and "hydropic" nuclear types were often present at once (Fig. 5). This fact, together with the identical cytoplasmic characteristics associated with the two types of nuclei, supports the belief that the nuclei, whether pyknotic or "hydropic," were of the same lineage.

Stellate Inclusions. No differences in size and shape could be detected between the stellate inclusions observed in this case and those commonly observed in giant cells in granulomatous inflammation, with

the possible exception that those in this case were on the average somewhat more perfectly formed (Figs. 7 and 8). Most of the inclusions stained moderately intensely with eosin, although an occasional one took up hematoxylin moderately well, a characteristic previously observed in the inclusions associated with other conditions.[2] They stained only faintly with either toluidine blue or crystal violet. With van Gieson's stain, they usually became yellow, although an occasional one displayed an orange tint. They were not colored by the Feulgen or the periodic acid-Schiff method, although they stained intensely with either acid or basic fuchsin (0.5 per cent solution in 50 per cent alcohol). In preparations stained by Dominici's method, the asteroid bodies were stained a delicate salmon color.

Of special interest is the reaction of the stellate inclusions to reagents for demonstrating proteins, since these reactions have not been previously reported. Addition of a drop of concentrated nitric acid to unstained sections processed through the various solvents down to water resulted in the development of a yellow color in the asteroid filaments. The bodies were not destroyed or dissolved after immersion in the nitric acid under a coverslip for 24 hours, although they underwent considerable distortion from the outset, due to movements of tissue after application of the acid.

The Thomas method for demonstrating arginine imparted a delicate pink color to the asteroid bodies, denoting a positive reaction (Fig. 9). As already noted, cytoplasmic granules also reacted positively, but this color could not be confused with color in the asteroid bodies since the latter were characteristically contained within colorless vacuoles.

The Millon reaction according to the method of Bensley and Gersh[9] resulted in a faint pink-orange coloration of the asteroid filaments. Again, the reaction was comparable to that observed in the cytoplasmic granules, which thus served as a positive control. Asteroid bodies in a non-caseous granuloma with clinical characteristics of Boeck's sarcoidosis gave results identical with those described previously with the xanthoproteic, Millon, and arginine reactions.

Of great interest among the staining reactions were the observations on preparations stained with Regaud's iron alum-hematoxylin, for this method gave maximal contrast to the asteroid bodies when desired (Fig. 10), or could be made to give negative results if destaining with mordant was carried to extremes (15 minutes or more). In this respect, the asteroid bodies reacted in a fashion similar to both nuclear material and cytoplasmic granules. Furthermore, in preparations destained for a given time, such as 5 minutes, some bodies would be

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sharply and completely stained, others would be only partially stained (Fig. 11) and still others would not be stained at all. In some cases, even though the radial filaments might be unstained, the central round core of the asteroid body would be black (Fig. 6), indicating that this core possessed a greater tenacity for the dye than did the other parts.

During study of the sections stained with Regaud's iron alum-hematoxylin, a possible relationship of the nuclei to the asteroid bodies suggested itself. This concept can be summarized best as follows: In the multinucleated myeloma cells, progressive alterations of nuclei are observed, as already described. To reiterate briefly, these changes consist in (1) enlargement of the nucleus; (2) enlargement and increase in prominence of the nucleolus, and (3) filling of the nucleus with non-staining material at the expense of chromatin until the nucleus consists of a vesicular globe with a prominent nucleolus, a rather thinned-out nuclear membrane, and only fine, apparently compressed fibrils of chromatin (Figs. 5 and 6).

The next step would appear to be rupture of the nuclear membrane. Nuclei actually undergoing this process were not observed; it is presumed to occur extremely rapidly. However, what is conceived to be the result of this process is the "fresh" asteroid, both filaments and core of which are stained deeply by Regaud's method (Fig. 10). The core apparently is represented by the nucleolus before nuclear rupture (Fig. 6), since it generally corresponds to this in size, shape, and central position. Furthermore, eccentrically placed cores are sometimes seen, corresponding to the occasional eccentrically located nucleolus (Fig. 10). The vacuole that characteristically surrounds the asteroid body appears to be derived from the non-staining material filling the nucleus before rupture.

The subsequent fate of the asteroid bodies appears to be as follows: The large vacuole surrounding the body subdivides in varying degree into a number of smaller vacuoles that at first are closely grouped but that later show some tendency toward dispersion. Filaments of the asteroid body tend to shorten and form irregular, somewhat spherical bodies that are contained within some of the vacuoles. These bodies are eosinophilic. The vacuoles and their contents at this stage are found most often somewhere near the periphery of the giant cell (Fig. 12). Eventually they are expelled from the cell, although actual observation of this act is rare (Figs. 12 and 13). The observation that the asteroid body by this time has disintegrated into a number of globular eosinophilic particles within numerous vacuoles accounts for the fact that extracellular asteroid bodies have not been seen.

It will be noted that the concept just presented is practically the reverse of that proposed by Jaques,[2] who supposed that multiple vacuoles containing eosinophilic bodies aggregate to form a single large vacuole, with the asteroid body forming therein from the eosinophilic bodies.

HISTOPATHOLOGIC OBSERVATIONS IN GAUCHER'S DISEASE (CASES 2 AND 3)

The histologic pictures in the spleens of these patients with Gaucher's disease were completely characteristic and will not be described at length. Greatly dilated sinusoids were filled with large multinucleated cells with voluminous cytoplasm that displayed the characteristic fibrillar striations of Gaucher's cells (Figs. 14 and 15). Imprints of the spleens stained by Wright's method likewise showed this fibrillar cytoplasmic pattern to advantage. In case 2, stellate inclusions could be found only rarely, and were generally rather imperfect, while phases of disintegration were frequent. Thus, one could easily find cells containing groups of "bubbles" in which small eosinophilic particles were present.

Stellate inclusions were numerous in case 3, it being not unusual to find two within a single giant cell (Fig. 14). They were not so numerous, however, as in the case of plasma cell myeloma. In both cases 2 and 3, the cytoplasm of the cells within which asteroid bodies were found displayed the same fibrillar structure as adjacent Gaucher's cells, leaving no reason to suppose that these cells were of different parentage than their neighbors. The fact that, according to current concepts, both Gaucher's cells and the giant cells of granulomatous reaction may be derived from histiocytes makes it difficult to define a giant Gaucher's cell and to differentiate it from a reactive giant cell that has phagocytized cytoplasmic "sheddings" from Gaucher's cells. Such distinction might, in fact, appear highly artificial at present, since the fundamental mechanism by which cerebrosides come to be present in Gaucher's cells has not been adequately demonstrated.

With the exception of the periodic acid-Schiff reaction, special staining and histochemical procedures were not performed on the stellate inclusions in the cases of Gaucher's disease described. It is anticipated that this report will provide a stimulus to further histochemical investigation.

COMMENT

The occurrence of stellate inclusions in Gaucher's disease perhaps may be interpreted properly as evidence supporting the concept, pro-

posed by others, that these structures in some way are related to disorders of lipid metabolism. However, it does not appear appropriate to conclude, on the basis of the present study, or previous studies, that asteroid bodies are composed entirely or even in part of lipids. It must be recalled that the proposal of Jaques[2] concerning the phospholipoprotein nature of these bodies is based predominantly on a positive Lorrain Smith-Dietrich reaction in spite of the fact that the solubility characteristics of asteroid bodies (insoluble in pyridine or a 1:1 mixture of xylene and glacial acetic acid) are not in accord with those of phospholipids. It would be of interest to determine if the positive Lorrain Smith-Dietrich reaction can be prevented by pretreatment with the solvents just mentioned, suggesting the presence of a surface coating of phospholipid.

On the contrary, the observations reported in this study might be interpreted as pointing to the importance of cerebrosides, rather than of phospholipids, in the genesis of asteroid bodies. What relation could cerebrosides have had in the formation of asteroid bodies in the myeloma cells in case 1? In view of incomplete histochemical study of the cells in this case, it is not possible to answer this question. It is perhaps pertinent, however, that the cytoplasm of these cells contained large amounts of material giving a positive periodic acid-Schiff reaction similar to that demonstrable in Gaucher's cells. This material might be kerasin or another cerebroside, since Morrison and Hack[13] have demonstrated this reaction to be positive in the cytoplasmic material of Gaucher's cells and in purified kerasin. The existence of a wide variety of chemical compounds that give positive periodic acid-Schiff's reactions does not, of course, permit any conclusion in this respect.

Positive reactions for protein as observed in asteroid bodies in the present study add to previous indirect evidence that such bodies are composed, at least in part, of protein. Cunningham's[1] study showed that the melting point of these bodies was not compatible with any known lipid. Jaques'[2] observations on solubilities were interpreted as suggesting a protein-lipid linkage. All attempts to demonstrate carbohydrates or minerals in inorganic state have failed. In the evaluation of the significance of positive reactions for protein in the present work, the ever present possibility of "false" reactions due to surface adsorption of protein must be recognized.[1]

As Cunningham[1] has pointed out, the occurrence of stellate inclusions in widely diversified reactions is strong evidence against these structures being specific for any disease. The present observations add

weight to that opinion. A common denominator influencing the formation of these bodies would appear more likely to be found within the asteroid-bearing cells than in the humoral chemical features of a disease process. The observations regarding sequential nuclear changes in case 1 suggest that such changes, followed by rupture, may be requisite to the formation of asteroid bodies. The frequency of occurrence of asteroid bodies in lipidic disorders needs only imply that intracytoplasmic lipids function as co-factors in their formation. Such a concept is not far removed from that suggested by Engle,[4] namely, that intracellular lipids may act as a template for the formation of asteroid bodies. Kopac[14] has presented evidence that lipids are capable of denaturing nuclear protein released by rupture of the nuclear membrane. In such a scheme, the template suggested by Engle would be on a molecular scale. A water-soluble polycerebroside recently isolated from spleens in Gaucher's disease by Uzman[15] may bear on this matter, as Uzman remarks on "the interesting properties which aqueous solutions of this material exhibit in terms of forming stable complexes with proteins in vitro."

Participation by nuclei in the formation of crystal-like bodies *in vivo* and *in vitro* is not unknown. In tissue culture radial bodies not unlike those under consideration have been observed and photographed in the process of formation during cellular breakdown.[16] Evidence is strong that Charcot-Leyden crystals are formed in part, at least, by components of the nuclei of eosinophils.[17-20] Charcot-Leyden crystals have been shown, furthermore, to give reactions characteristic of protein.[19] Their formation *in vitro* can be facilitated by the addition of detergents to suspensions of leukocytes.[17] Judging from photographs, it appears unlikely that the structures produced *in vivo* after injections of lipids by Hirsch[3] were identical with the asteroid bodies concerned here.

I have made preliminary attempts to bring about formation of asteroid bodies *in vitro* by incubating slices of fresh tuberculous renal tissue for 24 to 48 hours in detergent solutions and in sebaceous material obtained from a dermoid cyst. Although asteroid bodies were found in giant cells in such preparations, it was not possible to make a quantitative assessment because of natural variations in the control material. Further experiments along this line are indicated, using a spectrum of lipids, including the glycolipid described by Uzman[15] and surface-active agents.

That stellate inclusions are not artifacts resulting from chemical fixation has been ascertained through observation of these bodies in

fresh frozen sections of tissue showing a granulomatous reaction around an epidermoid cyst (Fig. 16).

Summary

Stellate cytoplasmic inclusion bodies have been observed within multinucleated myeloma cells in a lymph node in a case of somewhat atypical plasma cell myeloma, and within multinucleated "Gaucher's cells" in 2 cases of Gaucher's disease. These stellate inclusions were morphologically identical with inclusions commonly seen in giant cells of granulomatous reactions.

The asteroid bodies in the case of plasma cell myeloma gave positive xanthoproteic, Millon, and arginine reactions, indicating the presence of a protein component, although the possibility of adsorption artifacts was not excluded. Asteroid bodies in a case of non-caseating granuloma with a clinical picture of Boeck's sarcoidosis gave identical reactions.

The progression of nuclear changes noted in the case of plasma cell myeloma suggests a concept as to the formation of stellate inclusions. It is postulated that the inclusions are composed, at least in part, of nuclear material released by rupture of the nucleus. The presence of lipids or lipoproteins in the cytoplasm of giant cells may favor the formation of asteroid bodies by facilitating nuclear rupture or by physicochemical effects on nuclear components subsequent to nuclear rupture. The observations reported are considered to be further evidence in support of the view that stellate inclusions are not specific for any disease.

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[*Illustrations follow*]

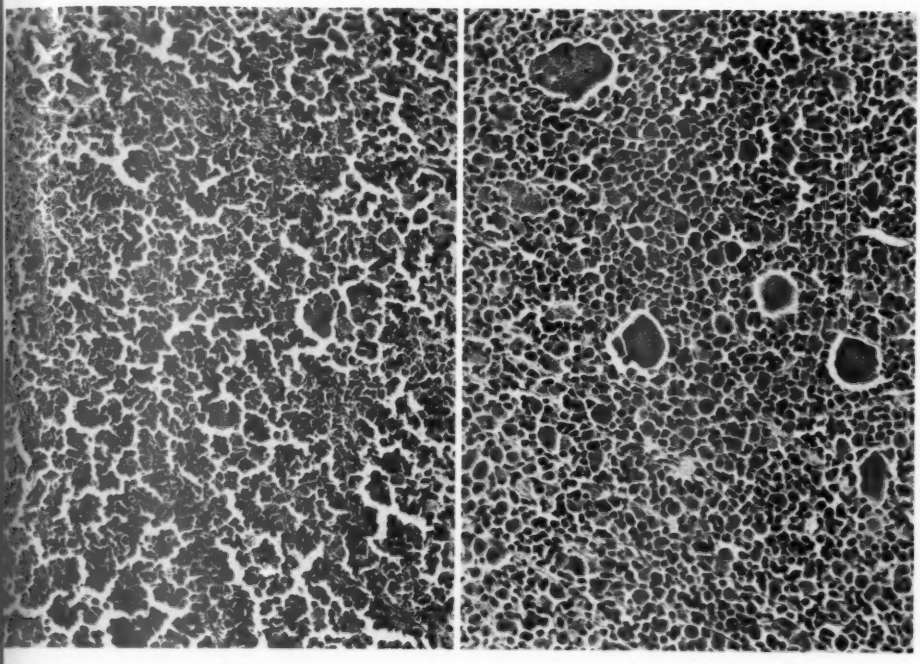
LEGENDS FOR FIGURES

FIG. 1. Multiple myeloma in lymph node (case 1). There is a predominance of immature plasma cells in this field; an occasional cell with voluminous cytoplasm is present. Hematoxylin and eosin stain. × 135.

FIG. 2. Multiple myeloma in lymph node (case 1). Transition from immature plasma cells to multinucleated giant myeloma cells is shown. Hematoxylin and eosin stain. × 135.

FIG. 3. Multiple myeloma in lymph node (case 1). The field is dominated by giant multinucleated myeloma cells, one of which contains more than 100 nuclei in the portion of the cell shown. Hematoxylin and eosin stain. × 135.

FIG. 4. Higher magnification of a portion of the field shown in Figure 1; of note are the plasma cell characteristics in some cells, with transition toward macrophage-like cells with abundant granular cytoplasm. Hematoxylin and eosin stain. × 640.



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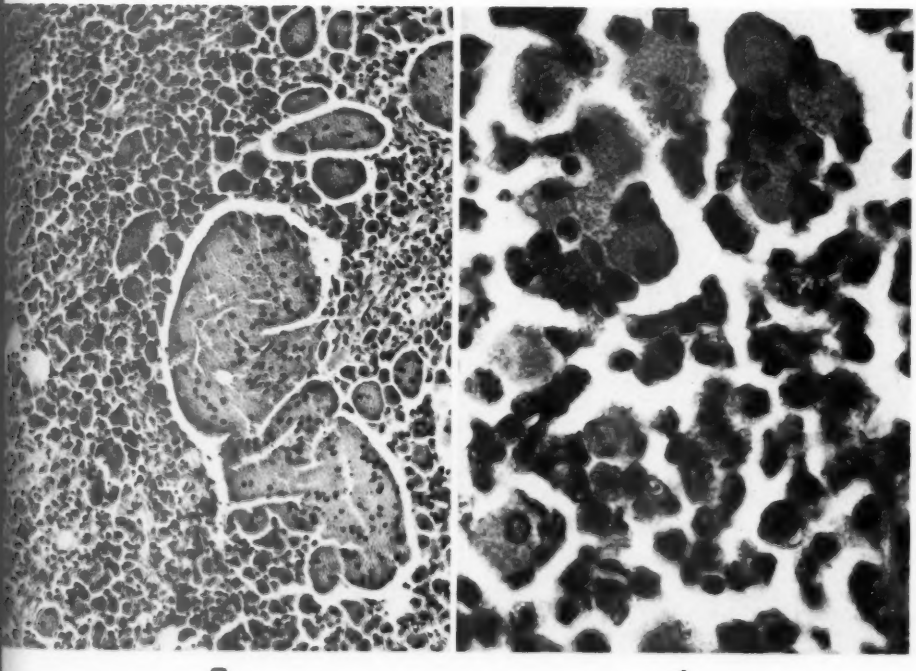

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FIG. 5. Multinucleated cell (case 1) showing nuclei in various stages of transition from small, compact type to swollen, "hydropic" type, presumably antecedent to asteroid formation. Regaud's iron alum-hematoxylin, destained in mordant for 15 minutes. × 610.

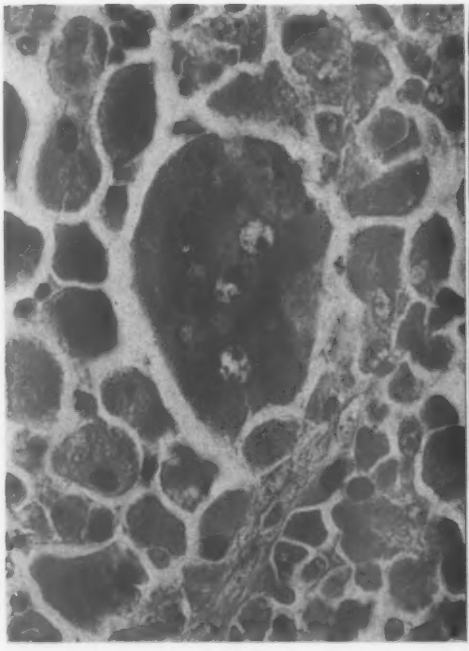
FIG. 6. Multinucleated cell (case 1) with asteroid body in lower right. The spines, or filaments, are unstained, while the central granule or core is black. Size, position, and staining of the central core correspond to nucleoli of nuclei at the opposite side of the giant cell. One of the latter nuclei is greatly swollen; the nucleolus is out of focus in this plane but is visible near the center of the nucleus. Rupture of this nucleus might result in formation of an asteroid body. Regaud's iron alum-hematoxylin, destained in mordant for 5 minutes. × 760.

FIG. 7. Asteroid body in giant cell in non-caseating granuloma presenting the clinical picture of Boeck's sarcoidosis. Hematoxylin and eosin stain. × 760.

FIG. 8. Asteroid body (case 1). Rather perfectly formed bodies of this type were common in this case. Hematoxylin and eosin stain. × 760.

FIG. 9. Case 1. Asteroid body showing positive reaction with T[illegible]mas' arginine method; filaments stain pink, as do cytoplasmic granules. × 1000.

FIG. 10. Case 1. Asymmetric stellate inclusion in giant cell; such an eccentrically placed core corresponds to an eccentrically placed nucleolus prior to nuclear rupture. All filaments are heavily stained. Regaud's iron alum-hematoxylin, destained in mordant for 5 minutes. × 760.



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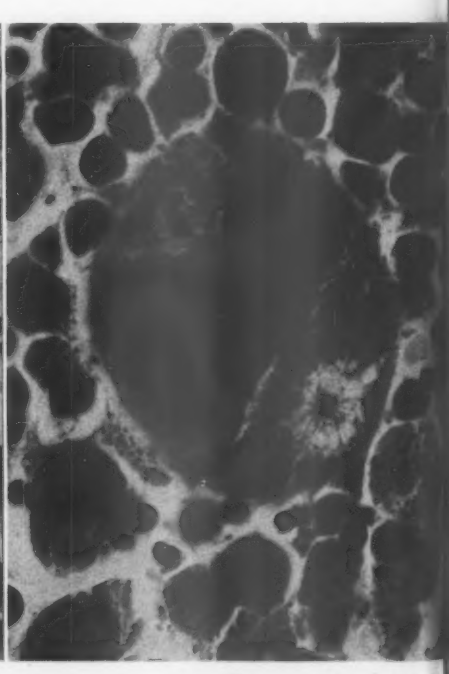

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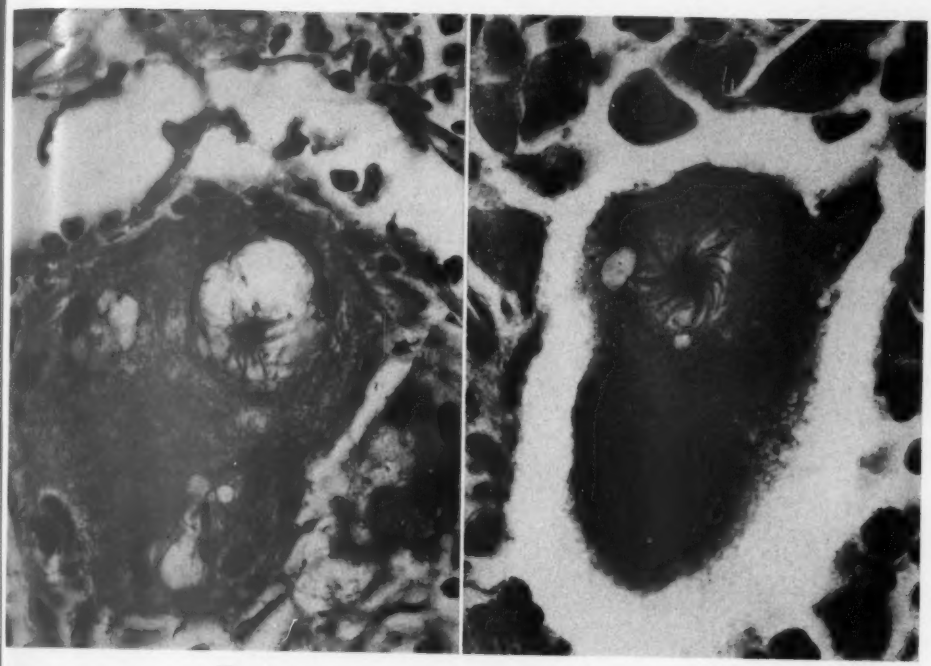

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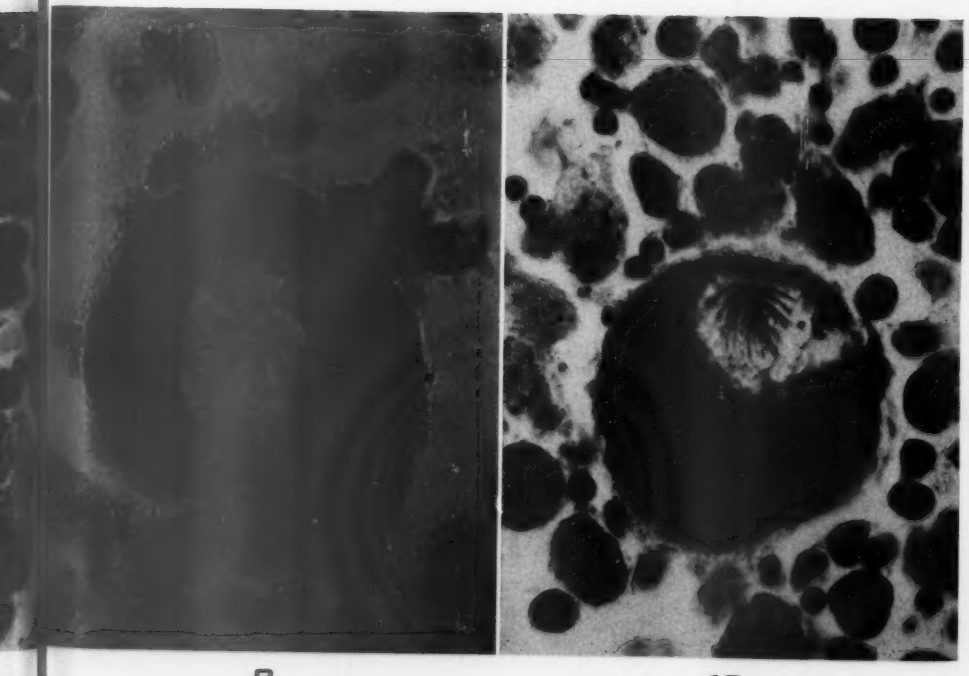

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FIG. 11. Case 1. Symmetric stellate inclusion in giant cell. Of note is the staining of the central core and only a few of the filaments. Regaud's iron alum-hematoxylin, destained in mordant for 5 minutes. × 760.

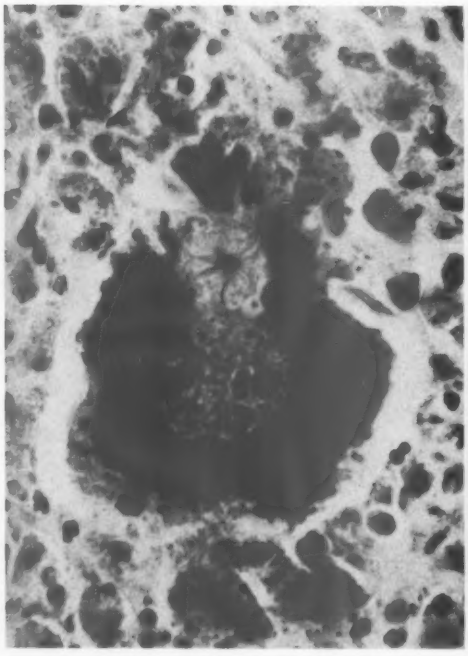
FIG. 12. Case 1. Remains of an asteroid body and the surrounding clear substance about to be expelled or released from a giant cell. Spines of the asteroid body are visible on the right side of the "vacuolated" area. Hematoxylin and eosin stain. × 760.

FIG. 13. Clear substance from the area around an asteroid body (case 1) subsequent to release from the cell, appearing as "bubbles" on the periphery of the cell. Periodic acid-Schiff's stain with hematoxylin counterstain. × 760.

FIG. 14. Two asteroid bodies in Gaucher's cell (case 3). These are well formed and are representative of those present in this case. Hematoxylin and eosin stain. × 760.

FIG. 15. Spleen in case 3 showing dilated sinuses choked with Gaucher's cells; in the upper left are the two asteroid bodies shown in Figure 14. Hematoxylin and eosin stain. × 135.

FIG. 16. Two asteroid bodies as seen in fresh frozen sections of giant cell reaction around an epidermoid cyst. Terry's polychrome methylene blue stain. × 1000.



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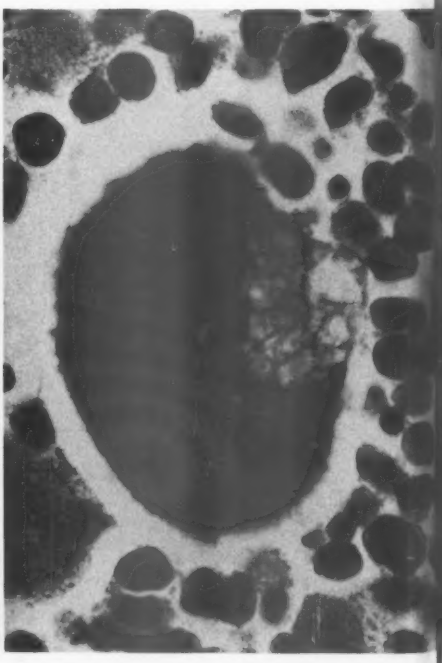

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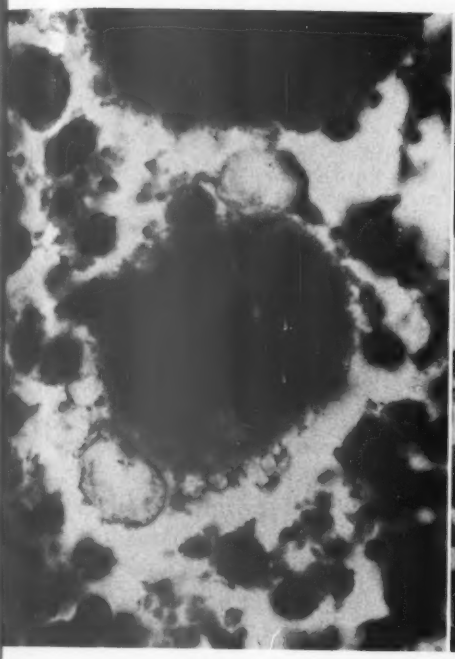

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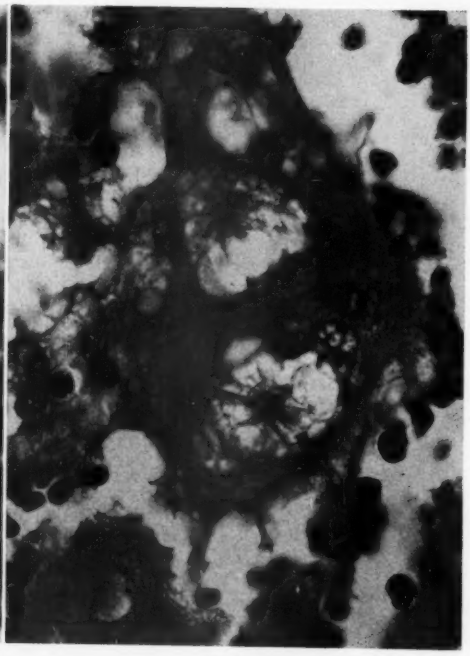

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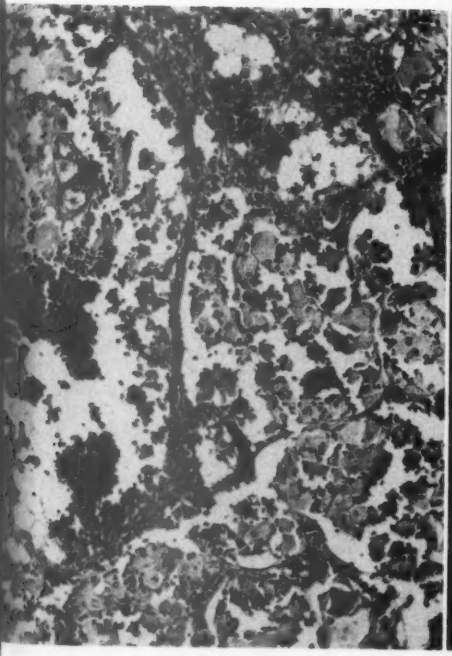

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16

THREE TYPES OF CHROMOPHIL CELLS OF THE ADENOHYPOPHYSIS

Demonstrated by a Modification of the Periodic Acid-Schiff Technique *

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The chemical composition of at least six hormones of the anterior hypophysis is fairly well established.[1] Growth hormone, adrenocorticotrophic hormone, and prolactin are simple proteins, whereas the gonadotrophins (F.S.H. and L.H.) and thyrotrophin are said to be mucoproteins containing appreciable amounts of carbohydrate. These latter hormones or their precursors should, therefore, be demonstrable by histochemical techniques that are specific for carbohydrates.[2]

The periodic acid-Schiff reaction was introduced independently by McManus[3] and Hotchkiss[4] as a histochemical method that resulted in the staining of carbohydrates in fixed tissues. The exact mechanism of this reaction is still in doubt,[5] but there is general agreement that treatment with periodic acid oxidizes polysaccharides to polyaldehydes which, in turn, react with Schiff's reagent (fuchsin-sulfurous acid) to produce a red-colored compound.

Pearse[6] has done extensive work on the human and various animal pituitary bodies, using a modification of the periodic acid-Schiff (PAS) technique to localize the mucoprotein hormones. He found that none of the acidophilic granules contains any demonstrable carbohydrate. However, all the classical basophils and some of the cells that are chromophobes with techniques of the Mallory type (acid fuchsin-aniline or methyl blue) contain granules that stained red following the PAS reaction. Subsequently, he differentiated two types of mucoprotein particles which he named beta and gamma granules.[7] Both are PAS-positive but they can be distinguished by a variety of staining reactions; *e.g.*, the beta granule is gram-positive and stains red with tetrazonium after benzoylation, whereas the gamma granule does not give either of these reactions. Pearse suggested that the difference between them was largely physical. The beta granule was said to be in a more highly polymerized state and to possess a somewhat

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† Alfred Stengel Research Fellow, American College of Physicians, 1952–53.

greater affinity for the Schiff's reagent. The granules of the acidophils have long been referred to as alpha granules.

We have elaborated on the Pearse trichrome-PAS staining method by the introduction of methyl blue as a second counterstain. The PAS-methyl blue procedure is as follows:

Method

1. Decerate sections in xylol and hydrate through alcohols to water.
2. Treat for 5 minutes in 0.5% aqueous periodic acid.
3. Rinse well in distilled water.
4. Treat for 15 minutes in Schiff's reagent.
5. Follow with sulfurous acid rinses, allowing 3 minutes for each of three changes.
6. Wash in running tap water for 10 minutes.
7. Stain in 1% aqueous orange G (C.I. 27) for 30 seconds.
8. Treat with 5% phosphotungstic acid for 30 seconds.
9. Wash for 30 seconds in running tap water.
10. Stain for 1 minute in 1% aqueous methyl blue.
11. Wash out the excess methyl blue in running tap water.
12. Dehydrate sections through several changes of absolute alcohol.
13. Clear in two changes of xylol and mount in xylol-balsam.

Solutions.

1. 1% aqueous periodic acid.
2. Schiff's reagent, prepared in this laboratory according to Lillie's[8] directions:
   Add basic fuchsin (C.I. 677), 1 gm., and sodium metabisulfite, 1.9 gm., to 100 cc. of 0.15 N hydrochloric acid. Shake at intervals for 2 hours.
   Add 500 mg. fresh activated charcoal (Norite). Shake for 1 to 2 minutes. Filter twice. Solution is ready for immediate use.
   Store in refrigerator.
   Make up fresh solution every 2 to 3 weeks.
3. Sulfurous acid rinse—to make 1 liter:

10% sodium metabisulfite	60 cc.
N/1 hydrochloric acid	50 cc.
Distilled water	1000 cc.

4. 1% aqueous orange G (C.I. 27).
5. 1% aqueous methyl blue.

Material

About 100 human pituitary bodies obtained from routine necropsies were fixed for 48 hours in 10 per cent formol-saline solution, embedded in paraffin, and sectioned in the horizontal plane at a thickness of 4 μ. Adjacent sections were stained with hematoxylin and eosin, methyl blue and eosin (according to the technique of Mann), and with the PAS-methyl blue stain. Some horizontal sections of pituitary bodies of normal rats were examined in a similar fashion.

Results

Prior to the addition of the methyl blue, all the PAS-positive granules stained a shade of red. Upon counterstaining with methyl

blue, some of the red granules became purple, possibly because of a selective adsorption of the dye.* As in Pearse's method, the orange G stained alpha granules and other acidophilic substances a shade of yellow.

PAS staining should give no trouble after formalin fixation. The proper preparation and use of Schiff's reagent is discussed in the literature.[9] Staining of the alpha granules by orange G may be lost or obscured in two ways: first, as orange G is soluble in water, it may be extracted from the section by the rinsing in tap water that follows immersion in the orange G stain; second, the orange alpha granules may be overstained by prolonged immersion in the methyl blue counterstain, with the result that all contrast is lost. Loss of the orange G from the section is preventable by rinsing in slightly acidulated water (1 per cent acetic acid). Overstaining with methyl blue may be avoided by using a control section to determine a satisfactory time in the stain.

Methyl blue staining is probably the most critical step. The success of the stain lies in the degree of contrast achieved between the red and purple (beta and gamma) granules. It is likely that first attempts will produce overstained preparations. The desired tinctorial contrast can be secured by careful technique. It is important to be familiar with the potency of the methyl blue solution in current use. It may be necessary to prolong the staining time as the solution ages. Control sections are the only basis for predictable results and should be used routinely.

Phosphotungstic acid is an essential agent in the method. It "fixes" the orange G in the section and prevents complete extraction of this dye in the methyl blue solution.

The red beta granules and the purple gamma granules are largely confined to separate cells; in a few instances, both types were found within the cytoplasm of a single cell. Therefore, with this stain, it is possible to differentiate tinctorially three granular and one "non-granular"† cellular types. The cell with the orange-staining granules in Figure 1 is a classical acidophil.

* Another example of this phenomenon is the response of the granules of mast cells and beta cells in the aldehyde-fuchsin staining of pancreatic islets. With aldehyde-fuchsin alone, both types are purple; after counterstaining with dilute basic fuchsin the mast cell granules become red whereas the beta cell granules remain purple.

† Rinehart and Farquhar,[10] in a paper presented to the 11th Annual Meeting of the Electron Microscope Society of America, 1953, have shown by electron microscopy that anterior pituitary chromophobes usually contain enough granules to be placed in the category of acidophil or basophil cells. Such granules may be either too small or too few to be resolved using conventional staining procedures and light microscopes.

The red beta granules and the purple gamma granules are both PAS-positive. Unfortunately one cannot name the cell containing gamma granules the gamma cell since this term has already been used to designate the chromophobe or "chief" cell. There is no objection to calling the cell containing beta granules the beta cell since this is in accord with common usage. However, to minimize confusion, we have been using the terms PAS-red and PAS-purple for these cell types.

A comparison of adjacent sections of human pituitary bodies, one stained with a stain of classical Mallory type (which shows red acidophils and blue basophils) and the other with the PAS-methyl blue stain, confirmed Pearse's observations that some "Mallory chromophobes" contain abundant mucoprotein granules. In most instances these stain purple after methyl blue is added.

In the rat, only the classical basophils are PAS-positive. After methyl blue some of these stain red and others purple, indicating two types of mucoprotein granule (Fig. 2).

Discussion

In 1940, Romeis[11] differentiated two types of basophils in the hypophysis of several species, including the human and the rat, using a combination of resorcin fuchsin and azo-carmine. Employing the Greek letter nomenclature first suggested by Percival Bailey, he called *beta* cells those with granules that stain from brown to violet with the fuchsin dye. His *delta* cells were those basophils which failed to stain with resorcin fuchsin but which could be differentiated from the chromophobes or *gamma* cells by an aniline blue counterstain. Later, Halmi[12] used the more satisfactory aldehyde-fuchsin technique of Gomori[13] to distinguish these two classes of basophil cells in the pituitary body of the rat. Although histochemical significance was not claimed for these techniques, they do afford additional evidence of an increased number of cellular types.

Recently Purves and Griesbach[14] reported two types of PAS-positive cells in the pituitary body of the rat. These differ in regional distribution, affinity to blood vessels, shape and size of granule, and reaction to moderate variations in the level of circulating sex and thyroid hormones. In Purves and Griesbach's classification the "gonadotrophs" are coarsely granulated, oval cells located mainly around the periphery of the gland in proximity to blood vessels. They respond selectively to castration by hypertrophy and hyperplasia and eventually develop a signet ring appearance with coalescent vacuoles displacing the nucleus

to one side. These are the well known "castration cells." Moderate excess of estrogen produces regressive changes in this group of cells. They postulated that only the gonadotrophic hormones are produced by this particular cell type. Their "thyrotrophs" are centrally located polyhedral cells containing fine granules and responding selectively to moderate deficiency and excess of thyroid hormone. They are the source of "thyroidectomy cells" which resemble the castration cells in their development. Feeding excess thyroid hormone induces hypoplastic changes in these cells. From this they inferred that these cells produced only thyrotrophic hormone. Halmi[15] then noted that the aldehyde-fuchsin-positive beta cells are identical with the thyrotrophs and agreed that they probably were the source of thyrotrophic hormone.

With the PAS-methyl blue stain the cells corresponding to Purves and Griesbach's[14] thyrotrophs stain red, and those resembling gonadotrophs stain purple, as shown in Figure 2. As yet we have no evidence that the PAS-positive red and purple cells in the human pituitary body represent solely the source of thyrotrophic and gonadotrophic hormones respectively. On the contrary, observations of the pituitary body in cases of Cushing's syndrome and Addison's disease have shown that variations in adrenal function can influence both these cells.

In 1935, Crooke[16] described a hyaline change which develops in many of the basophils in cases of Cushing's syndrome. The PAS-methyl blue stain shows the hyaline change occurring only in the PAS-red cells (Fig. 3). This confirms Pearse's observation[17] that the granules remaining in the Crooke's cell are of the beta variety.

However, in Addison's disease the large, atypical, more or less lightly granulated, transitional basophils described by Crooke and Russell[18] stain purple with the PAS-methyl blue stain (Fig. 4). This is in agreement with Pearse's finding that these cells contain gamma granules only. The significance of these observations awaits further investigation.

Lillie[19] has introduced a modification of the McManus-Hotchkiss PAS procedure, that employs picric acid-methyl blue as a second counterstain. This method differentiates connective tissues (collagen, reticulum, and basement membrane) into two groups: one which retains the original PAS red color, and the other which turns a shade of blue following the picric acid-methyl blue counterstain. He applied this technique to various tissues, including the hypophysis. With the highest concentration of methyl blue used, 0.4 per cent (as opposed to the 1 per cent in our method), he reported that the cyanophil (basophil) cells retained the original PAS red color. We have applied the "allochrome" procedure, exactly as outlined by Lillie, to the human hypophysis and find that *all* the PAS-positive cells turn purple. The cause of this discrepancy is not apparent. However, the purple gamma granules, as demon-

strated by the PAS-methyl blue (1 per cent) technique, might justifiably be termed "allochroic" since they change their original color.

Summary

A modified PAS stain, employing methyl blue as a second counterstain, was used to study a series of human and rat pituitary bodies. It readily differentiates the two types of mucoprotein particle which Pearse has termed beta and gamma *granules*. In so doing it discloses three types of chromophilic cells in the human pituitary body: the acidophilic alpha cell and two carbohydrate-containing cells which we call PAS-red and PAS-purple cells. It also distinguishes tinctorially two types of PAS-positive cells in the pituitary body of the rat, which correspond with the thyrotrophs and gonadotrophs of Purves and Griesbach and the beta and delta *cells* of Romeis and of Halmi.

Crooke's hyaline cytoplasmic change of pituitary basophils, the characteristic cellular lesion of hyperfunction of the adrenal cortex, occurs only in PAS-red cells; whereas, in Addison's disease, the numerous lightly granulated, transitional basophils (Crooke-Russell cells) contain only PAS-purple granules.

We are indebted to Professor E. A. Linell for the human pituitary material used in this investigation. We also wish to thank Dr. W. G. B. Casselman, Professor W. S. Hartroft, and Professor J. D. Hamilton for their advice and encouragement.

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[*Illustrations follow*]

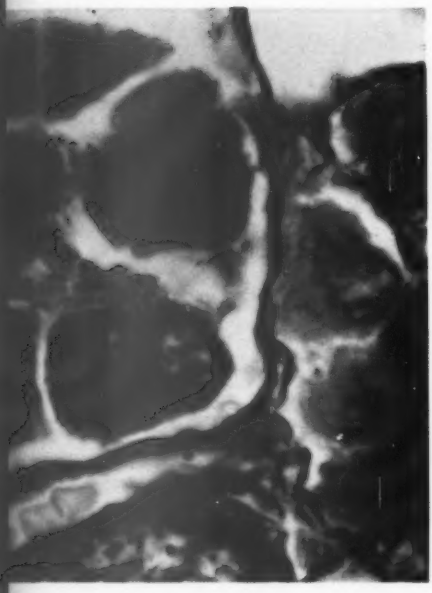
LEGENDS FOR FIGURES

FIG. 1. Normal human pituitary body. The three chromophils are shown: orange acidophil or alpha cell, on the right; PAS-red or beta cell, upper center; PAS-purple or delta cell, lower center. The PAS-red and PAS-purple cells contain mucoprotein granules. There is no chromophobe in this field. PAS-methyl blue stain. × 1640.

FIG. 2. Normal rat pituitary body: a PAS-red "thyrotroph," center right; and two PAS-purple "gonadotrophs" adjacent to a blood vessel, center and upper right. PAS-methyl blue stain. × 965.

FIG. 3. Human pituitary body: case of Cushing's syndrome with bilateral hyperplasia of the adrenal cortex. Crooke's hyaline change is seen only in the two PAS-red beta cells, center and upper left, which have only a few granules remaining. A PAS-purple delta cell is seen above an orange alpha cell, lower right. PAS-methyl blue stain. × 1640.

FIG. 4. Human pituitary body: case of Addison's disease due to atrophy of the adrenal glands. Increased in number and filling the field are the lightly granulated basophils described by Crooke and Russell.[18] These cells contain only PAS-purple granules and are considered to be modified delta cells. PAS-methyl blue stain. × 520.



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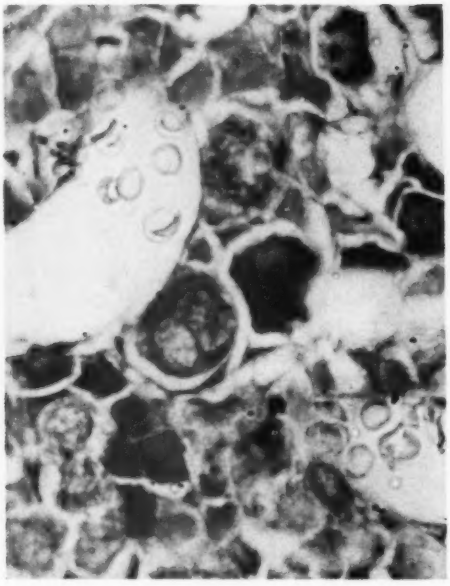

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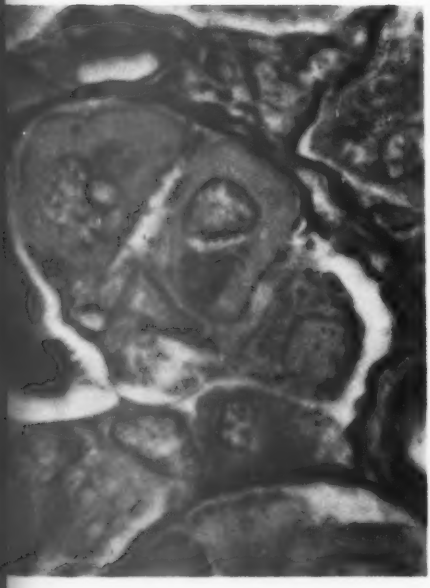

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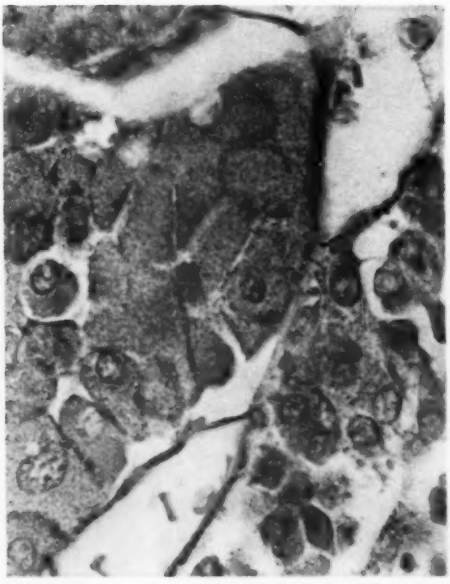

4

SUPPRESSION OF CORTISONE EFFECT ON REPAIR IN THE PRESENCE OF LOCAL BACTERIAL INFECTION *

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In studies on the modification by cortisone of inflammatory and reparative processes following experimentally produced injury, the usual inhibitory effect of cortisone was observed to be nullified by the accidental occurrence of local infection with suppuration.[1] It is the purpose of this report to describe the results of a series of experiments stemming from these observations, which appear to add further evidence to the hypothesis[1,2] that under the effect of cortisone, damaged tissues fail to release chemical substances necessary for the initiation and completion of repair.

EXPERIMENTAL FINDINGS

The experiments to be described were carried out on male albino rats weighing between 180 and 220 gm. The animals were treated intramuscularly with 15 mg. of cortisone acetate daily for 3 days before injury and daily thereafter until killed. The controls received daily injections of an equal volume of the suspending vehicle containing cholesterol. The original observations were made on several groups of cortisone-treated rats in which the tissue reactions to an irritant foreign body, in this case pledgets of gauze implanted subcutaneously, were studied. The technique of implantation of gauze pledgets (1 cm. square, 16-ply) has been described elsewhere.[3] Surgical asepsis was always observed. In the absence of infection, there is almost no reaction to a foreign body of this type in cortisone-treated rats.[2,4] When accidental infection occurred, the implanted foreign body was found at necropsy to be firmly adherent, surrounded by more or less abundant granulation tissue and purulent exudate. From one of these accidentally infected animals, a bacterial strain was isolated in pure culture early in 1953, and identified as a Gram-negative rod similar to *Actinobacillus actinomycetemcomitans*.

Using the same method, pledgets were implanted in a series of 16 rats, and a 72-hour broth culture of this bacterium was deliberately

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† Fellow of the Canadian Arthritis and Rheumatism Society.

injected into the gauze pledget, immediately after the implantation. At necropsy on the fifth day, the foreign body was in each case found to be surrounded by a zone of richly vascular granulation tissue, indicating that the cortisone was destroyed or its effect had been by-passed. (Broth alone caused no such effect.) This phenomenon was not related to the specific strain of organism since similar results were observed in a series of 6 rats when a 48-hour broth culture of *Staphylococcus aureus* obtained from an abscess in a human was injected into the pledget.

Recently, in reviewing a large number of histologic preparations from the experiments made by our group in the past several years, it was observed that practically each time that fibroplasia or angioplasia appeared in spite of adequate doses of cortisone, abundant polymorphonuclear leukocytic exudate was also present. For example, in 86 cortisone-treated rats with sterile pledgets of gauze implanted and killed 5 to 6 days after implantation, proliferation of definitely recognizable fibroblasts or fibroblasts and capillaries around the foreign body was observed in 26 instances. In all, there was purulent exudate also, sometimes even recorded in the protocols as a gross necropsy finding. This may be interpreted as suggestive that contamination and infection had occurred, which in turn had locally nullified the cortisone effect.

Some of our most striking and constant inhibitions of tissue response to injury by means of cortisone administration have been observed in 21 rats protected postoperatively against infection with daily injections of penicillin and streptomycin directly on the pledget (4,000 units of penicillin and 8 mg. of streptomycin daily).

The possibility exists that the organism or the products of its reaction in tissues might destroy the hormone *in situ*. The former possibility was explored in an experiment in which the Gram-negative rod similar to *Actinobacillus actinomycetemcomitans* was grown in broth containing a known quantity of hydrocortisone alcohol. There was no appreciable decrease in steroid concentration after 48 hours of growth.

Menkin[5] has reported a lack of cortisone effect in inflamed areas with an acid pH. While it is possible that local pH changes induced by bacterial growth may cause a heavy exudation of leukocytes even in the presence of cortisone, it is unlikely that the observed proliferation of granulation tissue can be explained on the same basis.

To explain our observations, recourse may be had to the admittedly vague but attractive theory that damaged tissues release locally chemical substances which are necessary for the completion of inflammation and repair.[6-10] Cortisone presumably blocks the sustained release of

these substances as soon as the acute injury has subsided and consequently interferes with the normal evolution of inflammation and repair. On the other hand, protracted tissue damage such as that caused locally by a bacterial infection could well bring about the continuous release of specific substances necessary for the continuation of the reparative processes. The suggestion is made that such a mechanism might explain the proliferation of granulation tissue obtained in cortisone-treated rats by combining the mechanical irritation of a foreign body with bacterial infection. Previous observations from this laboratory on the lack of cortisone effect in the early stages following injury may be interpreted in a similar manner.[2]

It is probable that many apparent discrepancies in the reported results of different investigators may be explainable on the basis of protracted tissue damage caused by infection or otherwise. Taubenhaus,[11] studying the effects of cortisone on turpentine abscesses, was able to reduce but not completely abolish the formation of granulation tissue. Zoger,[12] using the turpentine abscess technique in adrenalectomized, cortisone-treated rats, made similar observations. He also remarked that in the first 24 hours there was no difference between the cortisone groups and the controls.[12] The protracted tissue destruction caused by the chemical irritant might induce a more prolonged release of the presumed substances necessary for the reparative processes to take place. Taylor, Dittmer, and Porter,[13] studying the healing of open losses of substance in rats, were unable "to demonstrate significant faulty wound healing or decreased fibroblastic proliferation following the administration of cortisone." But these were open wounds, exposed to contamination and infection, and the same authors made the significant statement that "some of the sections which show the most pronounced fibroblastic response were seen in infected wounds of the cortisone group." This observation is in keeping with the results of our experiments, in which abundant granulation tissue was caused to appear in cortisone-treated rats by associating bacterial infection and an irritant foreign body, and, conversely, the inflammatory and reparative response was reduced to a minimum by protecting the animals with antibiotics.

Conclusions

The interpretation of the observations described in this report is in agreement with the theories[6,7,9,10] which suggest that the tissue responses to injury—inflammation and repair—are induced locally by chemical substances released, probably over a protracted period of time, by the injured tissues. In addition, it seems fair to postulate

that, after the effect of the injurious agent has subsided, under the influence of adequate doses of cortisone, the release of these substances is depressed or inhibited. Following a first phase of acute inflammation which is not influenced by the hormone,[2,12] the inflammatory and reparative processes are, so to speak, blocked. This inhibition in turn can be by-passed by processes such as a local infection through which tissue damage may be protracted.

We have made no appreciable progress toward the identification of the chemical nature of this substance or, more probably, substances. There is, however, some evidence[1,2,4,12] that at least some of them are acid mucopolysaccharides, in the absence of which proliferation of fibroblasts and endothelium does not take place. Experiments directed along these lines are continuing in our laboratories.

Summary

Cortisone-induced depression of repair can be nullified by spontaneous infection of experimental wounds.

Marked proliferation of granulation tissue in animals treated with adequate doses of cortisone was observed when the mechanical trauma of an irritant foreign body was combined with local bacterial infection.

Conversely, the most constant and most marked results in inhibiting inflammation and repair with cortisone were obtained in animals treated with daily injections of antibiotics.

It is postulated that the protracted tissue damage caused by the bacteria or their products brings about the release of specific substances necessary for the initiation and continuation of the reparative processes which would otherwise have been inhibited by cortisone.

We are indebted to Miss Daisy Mapes, R.N., for her efficient and intelligent assistance in all of the animal experimental work; to Miss Margaret J. Clute of the Department of Microbiology for the bacteriologic work; and to Drs. Joseph Jailer and Jay Gould for the determinations of 11-oxysteroids by a modification of the Porter-Silber method.[14]

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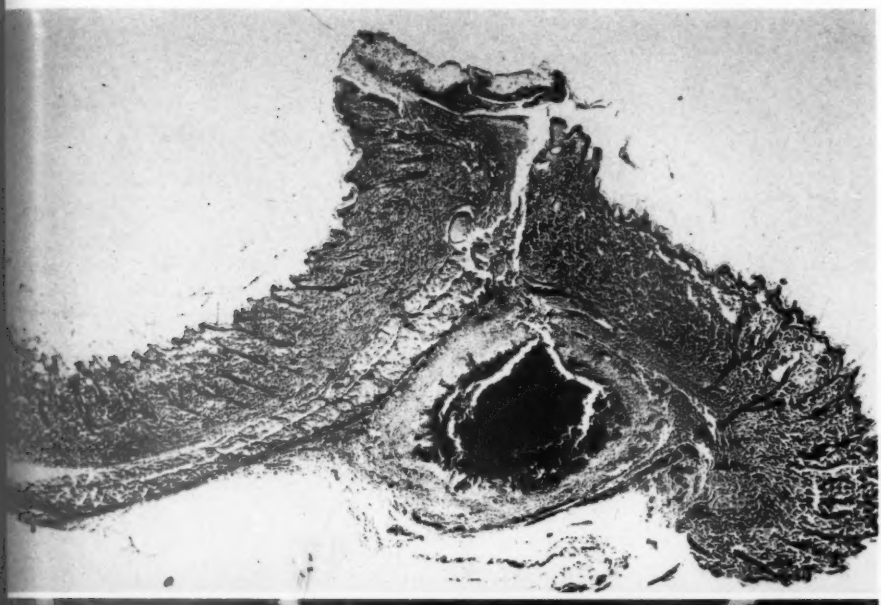
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. P. & S. 40000-A. Low-power photomicrograph of a sutured skin wound in a cortisone-treated rat 5 days postoperatively. The skin wound itself shows no repair, but due to accidental infection there is a subcutaneous abscess surrounded by a zone of granulation tissue. Hematoxylin and eosin stain. × 16.5.

FIG. 2. P. & S. 40000-A. Detail of Figure 1 at higher magnification. There is proliferation of fibroblasts and of capillary tufts in the zone surrounding the abscess. Hematoxylin and eosin stain. × 140.



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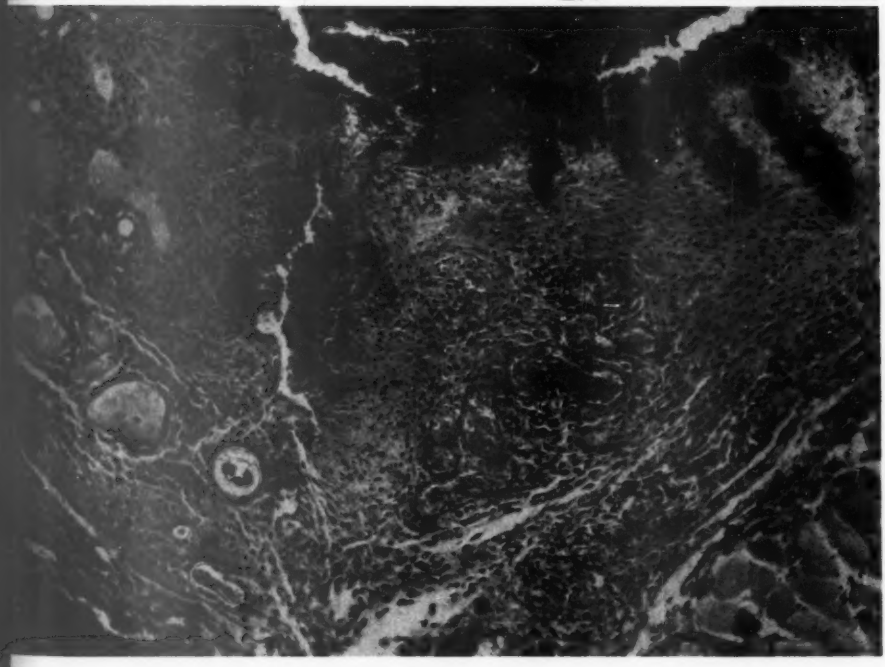

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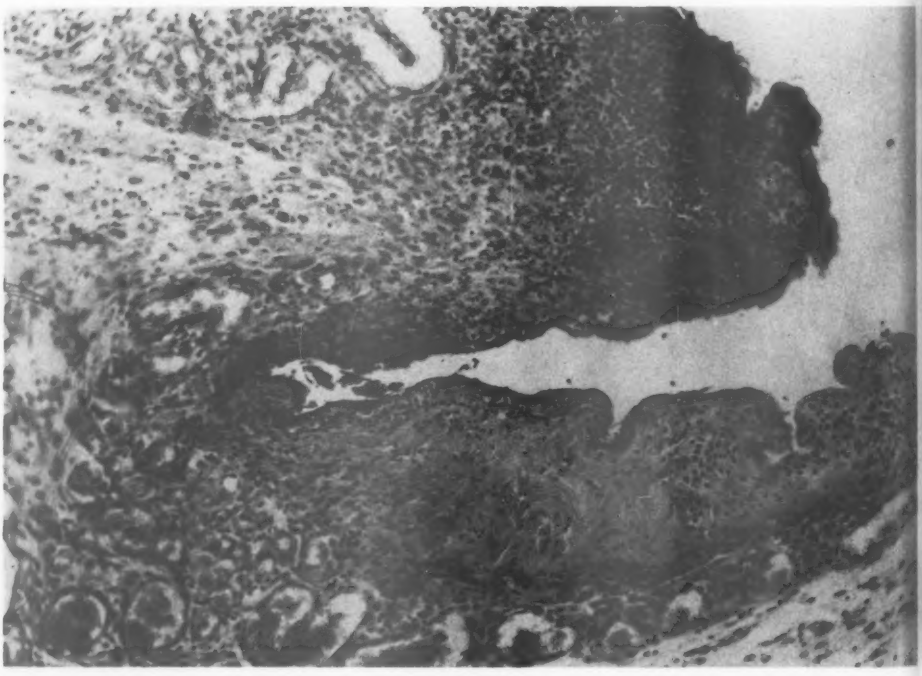
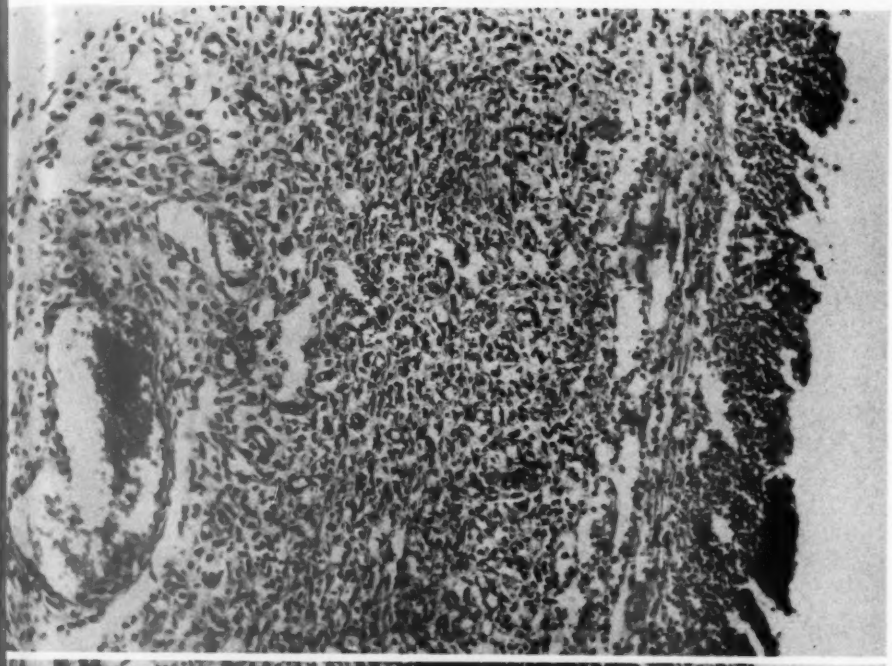
FIG. 3. P. & S. 40746-B3. Granulation tissue and purulent exudate surrounding an implanted pledget of gauze 5 days postoperatively in a cortisone-treated rat in which accidental infection occurred. The gauze was grossly adherent. Hematoxylin and eosin stain. × 140.

FIG. 4. P. & S. 41998-T2. Abundant granulation tissue and purulent exudate surrounding an implanted pledget of gauze in a cortisone-treated rat in which a bacterial suspension (rod allied to *Actinobacillus actinomycetemcomitans*) was also injected directly into the gauze. The foreign body was grossly adherent, encapsulated, and surrounded by exudate. Five days postoperatively. Hematoxylin and eosin stain. × 140.

FIG. 5. P. & S. 41998-T3. Findings similar to those shown in Figure 4, from another rat of the same experimental group. Hematoxylin and eosin stain. × 140.





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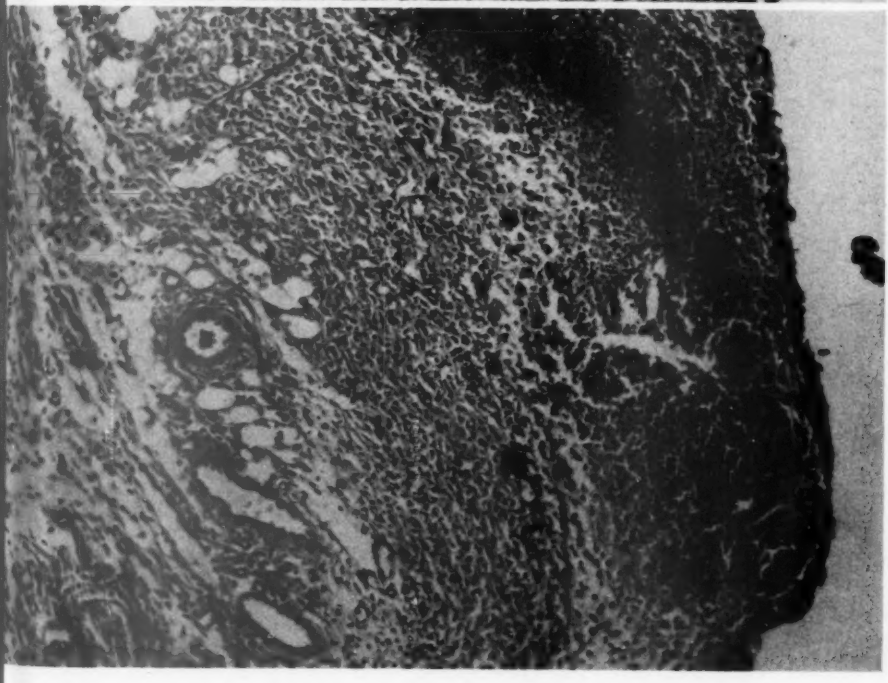

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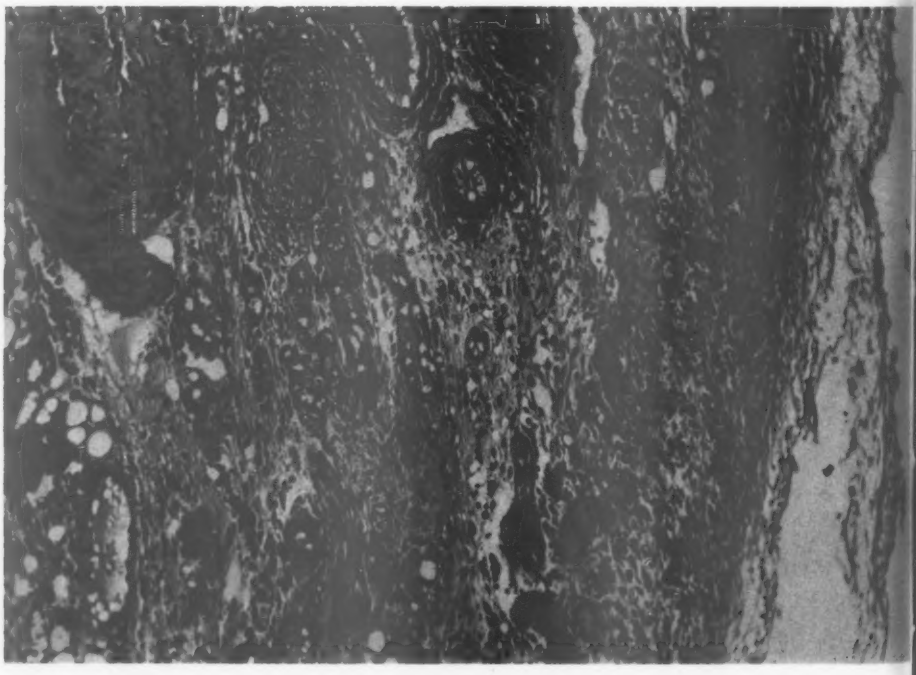
FIG. 6. P. & S. 43365-B. Abundant granulation tissue and fibrinous exudate surrounding an implanted pledget of gauze, 5 days postoperatively in a cortisone-treated rat. A bacterial suspension (*Staphylococcus aureus*) was injected in the gauze at the time of operation. At necropsy, the gauze was firmly adherent and encapsulated. Hematoxylin and eosin stain. × 140.

FIG. 7. P. & S. 40796. Highly cellular and vascular granulation tissue and purulent exudate in a control rat 5 days following implantation of gauze pledget and injection into gauze of a bacterial suspension (rod allied to *Actinobacillus actinomycetemcomitans*).

FIG. 8. P. & S. 41995-B3. Practically complete lack of inflammation or repair in the compressed connective tissue surrounding a pledget of gauze 5 days postoperatively in a cortisone-treated rat. This animal also received daily injections of antibiotics (see text). Hematoxylin and eosin stain. × 140.



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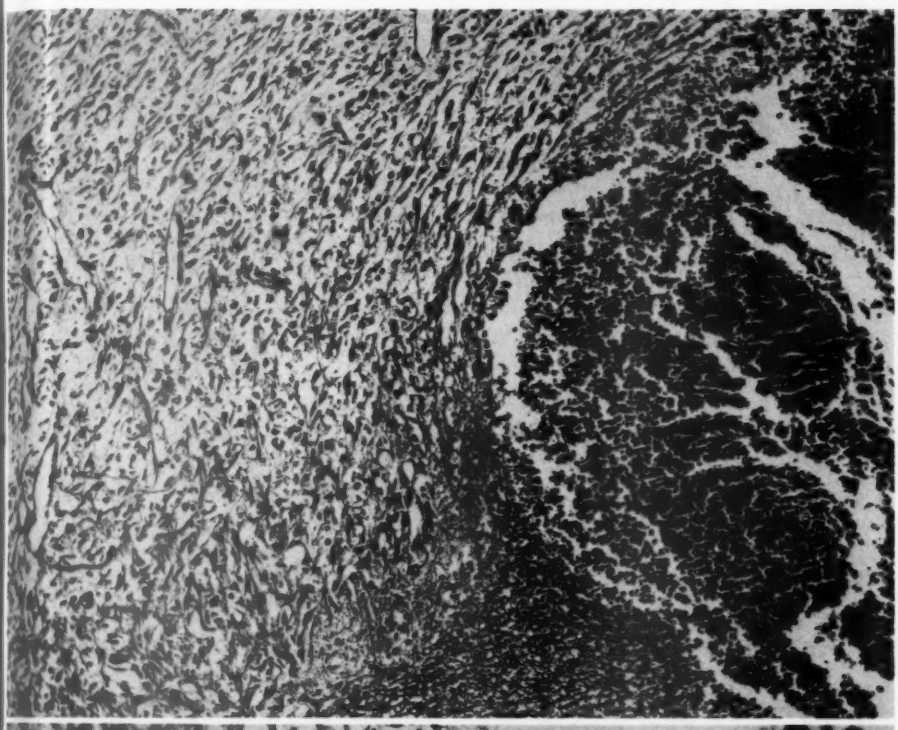

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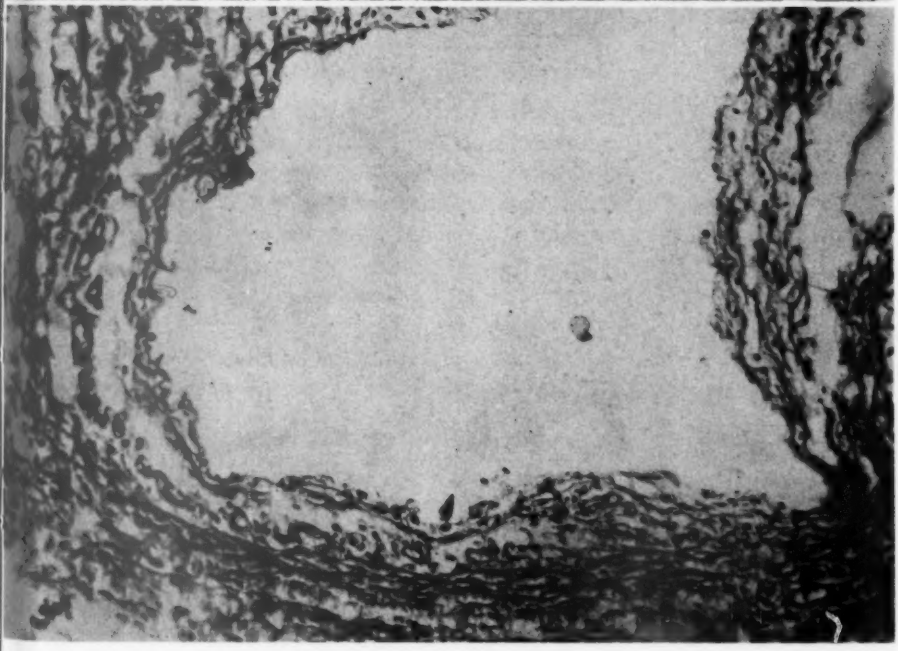

8

THE EFFECTS OF ADRENOCORTICOTROPHIC HORMONE AND CORTISONE UPON ACQUIRED IMMUNITY TO TRICHINOSIS IN MICE *

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In a recent article, we reported upon the unfavorable use of ACTH and cortisone with *Trichinella spiralis* infections in mice.[1] It was found that a slight increase in mortality occurred in animals given ACTH and that Trichinella-infected mice given cortisone treatment showed marked increase in morbidity and mortality as compared with untreated, infected animals. Because of the general interest in the relationship of adrenal cortical hormones to the immune mechanism, it was believed desirable to test the effects of ACTH and cortisone upon actively acquired immunity to challenge infections of *T. spiralis* in mice. A discussion of the rôle of adrenal cortical activity in relation to the immune response may be found in the review by Fischel.[2]

That acquired immunity to trichinosis may be conferred by a previous infection was demonstrated in the rat by Ducas[3] and McCoy[4] and in the mouse by Culbertson.[5] A high degree of acquired immunity to a challenge infection of Trichinella larvae may be demonstrated in mice actively immunized by a single sublethal active infection.[6] In the present study the degree of active immunity to challenge infections of *T. spiralis* was determined by the morbidity and mortality associated with the experimental infections.

MATERIALS AND METHODS

The animals used in this study were Swiss mice from our own colony. The stock strain of *T. spiralis* has been maintained in mice for several years. The mice were immunized when 4 to 6 weeks of age with active infections of Trichinella larvae. Challenge infections were given 4 to 6 weeks following the single immunizing infection. All animals were given infective doses on the basis of larvae per gram of body weight. Most of the procedures employed in the experimental infections were described in an earlier paper.[1]

ACTH (Armour) was diluted in 0.85 per cent saline solution, pH 7.1; cortisone acetate (Merck) was diluted in an aqueous vehicle†

* This research was done under the auspices of the Atomic Energy Commission.
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† We are indebted to Dr. Elmer Alpert, Medical Division, Merck & Co., Inc., Rahway, N.J., for a supply of aqueous vehicle no. 1.

control, and combiotic (Pfizer)* with 0.85 per cent saline solution. Injections were made subcutaneously in 0.1 ml. doses. The results from a series of experiments are discussed in terms of three basic experiments as follows:

1. Effects of ACTH upon acquired immunity
2. Effects of cortisone upon acquired immunity
3. Effects of cortisone and combiotic upon acquired immunity.

Experiment 1. Effects of ACTH upon Acquired Immunity

Experiment 1 was designed to test the effects of daily administration of 0.25 mg. of ACTH upon Trichinella-immune mice given a challenge dose of Trichinella larvae. The mice were immunized with a single infection of 160 to 200 larvae (10 larvae per gm.). Four weeks later, the immunized animals and non-immunized control animals were challenged with an infective dose of 1,500 larvae (75 larvae per gm.). An infective dose of 75 larvae per gm. usually constitutes an LD_{100} infection in our non-immunized mice 4 to 8 weeks old. Subcutaneous injection of 0.25 mg. of ACTH was started 1 day after infection and continued daily for 30 days.

The results of this experiment are shown in Table I. In the non-

Table I

Effects of ACTH upon Active Immunity to Trichinella spiralis Infection in Mice

Group description	No. of mice	No. which died	Per cent which survived
1. Trichinella challenge in non-immunized mice	79	76	3.8
2. Trichinella challenge in immunized mice*	69	11	84.1
3. Trichinella challenge in immunized mice treated with ACTH*	70	18	74.3

* Immunized with an active infection of Trichinella larvae (10 larvae per gm.); challenge infection (75 larvae per gm.).

immunized mice, group 1, the challenging infection closely approached an LD_{100}. A high degree of active immunity to the challenge infection may be noted for the immunized animals in group 2. ACTH treatment of the immunized mice, group 3, produced a slight but not significant increase in mortality.

* Charles Pfizer & Co., Brooklyn, N.Y.; combination of crystalline penicillin and dihydrostreptomycin.

Experiment 2. Effects of Cortisone upon Acquired Immunity

Active immunization of 6-weeks-old mice was accomplished by a single infection of 160 to 200 larvae (10 larvae per gm.). The challenging infective dose of 1,500 larvae was given 4 weeks after the single immunizing infection. Daily subcutaneous injection of 0.5 mg. of cortisone acetate was initiated 1 day post-infection and continued for 30 days.

As shown in Table II, none of the non-immunized mice in group 1

Table II

Effects of Cortisone upon Active Immunity to Trichinella spiralis Infection in Mice

Group description	No. of mice	No. which died	Per cent which survived
1. Trichinella challenge in non-immunized mice	79	79	0
2. Trichinella challenge in immunized mice*	74	8	89.2
3. Trichinella challenge in immunized mice treated with cortisone*	76	65	14.5

* Immunized with an active infection of Trichinella larvae (10 larvae per gm.); challenge infection (75 larvae per gm.).

survived the challenge infection. The actively immunized mice, group 2, demonstrated a high degree of active immunity to the challenge with 89 per cent of the animals surviving the infection. Cortisone treatment of the immunized and challenged animals, group 3, produced a significant breakdown of their actively acquired immunity to re-infection with Trichinella larvae. Of these group 3 animals, 75 per cent less survived the Trichinella challenge than of the untreated animals of group 2.

Pathologic Findings. Histologic examination was made of sections of various organs from mice treated as follows: (1) Trichinella-immunized, (2) Trichinella-immunized plus cortisone, (3) Trichinella-immunized plus a challenge infection, and (4) Trichinella-immune plus a challenge infection and cortisone. One mouse from each of these groups was sacrificed on the 5th, 9th, 21st, and 22nd day, and some were sacrificed on the 27th day of the infection, when available.

Study of the intestines revealed no ulceration or inflammatory reaction about the burrowed gravid worms in the challenged groups.

Microscopic examination of striated muscles revealed a difference

in mice having had a challenge infection (groups 3 and 4) compared to those without the challenge infection (groups 1 and 2), so that the major portion of the slides could be distinguished when examined as unknowns. This was possible largely because of the presence of young larvae and prominence of the reactive muscle nuclei in the challenge groups.

An effort was made to distinguish the muscles of groups 3 and 4. This was possible in some on the basis of the intensity of the acute inflammatory exudate; it was somewhat less in some of the cortisone-treated group than in the untreated group. This difference was not evident in all and where discernible, was slight. There appeared to be no difference in the number of larvae in the muscle, although direct larval counts were not performed.

Experiment 3. Effects of Cortisone and Combiotic upon Acquired Immunity

Several papers[7,8] have been concerned with spontaneous bacterial infections in animals given extended treatment with cortisone. Frothingham[9] and Gursch[10] reported extensive damage to the intestinal mucosa and villi in rats by maturing and adult trichinae, which damage presents an opportunity for bacterial organisms from the digestive tract to enter the lymph channels and blood of the host. In view of these reports, a bacteremia by enteric organisms normally found in the gastro-intestinal tract could be responsible for the breakdown of active immunity observed in cortisone-treated mice in the preceding experiment.

An antibiotic control experiment was carried out to test the effects of combiotic (Pfizer), and cortisone combined with combiotic, upon acquired immunity to re-infection with *T. spiralis*. Subcutaneous injection of combiotic and 0.5 mg. of cortisone was initiated 1 day after infection and continued daily for 30 days. The combiotic was given in 0.1 ml. injections and contained 2,000 units of crystalline penicillin and 2,500 μg. of dihydrostreptomycin sulfate. Mice 4 weeks old were immunized with a Trichinella infection of 10 larvae per gm. and challenged 4 weeks later with an infective dose of 65 larvae per gm.

As shown in Table III, the challenge infection in non-immunized mice (group 1) killed about 75 per cent of the animals. The immunized mice in group 2 demonstrated a high degree of active immunity with 90 per cent of the animals surviving the challenge. As found in experiment 2, the active immunity of the immunized, challenged, and cortisone-treated mice in group 3 was significantly lowered. The com-

bined treatment of cortisone and combiotic (group 4) did not significantly alter the survival above that observed for the cortisone-treated mice in group 3. There was no difference in the survival of immunized, challenged, and combiotic-treated mice (group 5) compared with the immunized and challenged control (group 2). From these data, it appears that the loss of actively acquired immunity to trichinosis in mice by extended treatment with 0.5 mg. of cortisone is not caused by

TABLE III

Effects of Cortisone and Combiotic upon Active Immunity to Trichinella spiralis Infection in Mice

Group description	No. of mice	No. which died	Per cent which survived
1. Trichinella challenge in non-immunized mice	30	22	26.7
2. Trichinella challenge in immunized mice*	30	3	90.0
3. Trichinella challenge in immunized mice treated with cortisone*	32	16	50.0
4. Trichinella challenge in immunized mice treated with cortisone and combiotic*	32	14	56.3
5. Trichinella challenge in immunized mice treated with combiotic*	32	3	90.6

* Immunized with an active infection of Trichinella larvae (10 larvae per gm.); challenge infection (65 larvae per gm.).

spontaneous bacterial infection or secondary bacterial invasion of organisms from the gastro-intestinal tract. This is further substantiated by histologic studies which showed no significant difference in the inflammatory reaction in the intestines of cortisone-treated and untreated infected mice.

Weinstein[11] studied the effect of cortisone upon the acquired immune response in white rats challenged by exposure of shaved belly skin to *Nippostrongylus muris* larvae. Rats given 2 mg. of cortisone daily 5 days prior to the challenge and for 11 days until necropsy showed almost complete suppression of cellular response in the skin. Untreated rats showed an intense inflammatory response in the skin. A greater number of worms was recovered from the cortisone-treated animals; this effect appeared to be related to the suppressed cellular response. Baughn[12] found that adrenalectomy of Trichinella-immunized mice increased natural resistance and acquired resistance to *T. spiralis*. The adrenalectomized mice were maintained with desoxycorticosterone acetate. A reduction in the number of adult worms was

observed in adrenalectomized mice. In the present study no attempt was made to compare adult or larval worm burden in cortisone-treated and untreated mice.

Summary

Cortisone treatment of mice immunized by active infection of *Trichinella spiralis* and challenged with Trichinella larvae produced a significant breakdown of their acquired immunity to re-infection. Daily administration of ACTH to immunized and challenged mice effected a slight but not significant increase in mortality rate over that observed in untreated control animals.

Combined treatment of immunized and challenged mice with antibiotics and cortisone failed to prevent the depressing effect of cortisone upon acquired immunity to Trichinella infection. Pathologic study revealed no significant ulceration or inflammatory reaction about the adult worms in the intestine of cortisone-treated and untreated animals.

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ULTRAVIOLET MICROSCOPY OF GLOMERULAR DISEASES *

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A modern concept of the glomerulus and its pathology is being developed by the applications of histochemistry, phase microscopy, and other newer morphologic methods. The changes of renal disease are now being regarded as more like pathologic processes elsewhere in the body and less like the reactions of an organ apart. In support of this general trend may be appended the following report of exploratory investigations of glomeruli, undertaken with the Polaroid color-translating ultraviolet microscope.

The general objective at the onset of the study was to determine whether stromal tissues had any peculiarities of ultraviolet absorption which would allow distinguishing abnormal from normal connective tissue. Little information on this subject is found in the literature. Glomeruli were chosen because by microscopic examination with visible light of unstained preparations on the Polaroid ultraviolet microscope stage, it was possible to recognize these structures easily and to orient them for ultraviolet photomicrography. Later, when the slides had been stained by routine or histochemical methods, it was feasible to identify and study the same glomeruli. Also a number of different human glomerular diseases with available pathologic material could be assembled readily.

The surprising finding that each major glomerular disease entity had a different ultraviolet absorption behavior, at times distinctive or diagnostic, was quite unanticipated. On this account, and because of the theoretic aspects, a report seemed desirable, although based only on a limited number of cases.

MATERIALS AND METHODS

Fresh kidney tissues, mostly from human surgical and necropsy specimens, but occasionally from rats or hamsters, were processed by freezing-drying and embedded in paraffin *in vacuo* with the method and

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apparatus previously reported.[1] Microscopic sections were dry-cut and mounted on Vycor slides by a technique originally devised for autoradiography.[2] It was necessary to have sections less than 4 μ thick for satisfactory ultraviolet microscopy. Sections were mounted in glycerin and covered with Vycor coverglasses rimmed with paraffin. Unavailability of fresh specimens of some less common kidney diseases required the use of blocks fixed in Zenker's solution, processed by the Autotechnicon, and embedded in paraffin for part of the study. From these, sections were cut and mounted as has been described.

The design and operation of the Polaroid color-translating ultraviolet microscope have been reported,[3,4] as well as the results of its use in some cytologic investigations.[5,6] The machine allows selection and sequential photomicrography, on 35 mm. film, at any three chosen ultraviolet wave-lengths in the range 235 to 400 mμ (2350 to 4000 Ångström units), of a microscopic field perfectly focused. The three separate photographic images are developed rapidly and projected superimposed as one picture on a 6-inch screen, translated into the colors blue, green, and red. A brilliantly colorful picture is seen for many tissues, due to their ultraviolet absorptive properties. It has been possible to obtain 40 to 50 sets of three pictures in 1 day.

For the purposes of this study the color blue was used for the longest wave-length chosen, green for the intermediate, and red for the shortest wave-length of each set of three. In general each individual field was studied at three different sets of wave-length combinations, as follows:

	blue	*green*	*red*
1st set	280 mμ	263 mμ	240 mμ
2nd set	280 mμ	263 mμ	248 mμ
3rd set	248 mμ	240 mμ	235 mμ

The sets employed allowed the estimation of absorptive behavior characteristics over the range 280 to 235 mμ. Densitometry studies of the permanent film records would allow the plotting of absorption curves, but this has been considered undesirable in the early stage of the development of pathologic ultraviolet microscopy.

Approximately 20 different cases were investigated. In each case more than one area and usually at least two different slides were studied. From two to six different glomerular fields were photographed for later interpretation (Table I). Additional fields photographed which proved unsatisfactory for reasons of section thickness, residual paraffin on slides, or other technical reasons are not included. A number of fields were photographed also to study arterioles and arteries,

and this material is not included in the present report. About 8½ days of operation of the Polaroid microscope were required for these two types of study, or an estimated 24 hours of actual operating time to obtain pictures of the 56 glomerular fields for interpretation.

For detailed study and comparison, black and white photographic prints, review of projected film strips, and accurate water-color repro-

TABLE I

Color-Translated Ultraviolet Absorptions of Glomerular Stroma

	Cases	Fields	1st Set (280, 263, 240 mμ)	2nd Set (280, 263, 248 mμ)	3rd Set (248, 240, 235 mμ)
Normal					
Frozen-dried*	2	4	Red-violet†	Gray-violet	Gray-tan
Zenker's	2	4	Red-violet	Pink-violet	Gray-violet-tan
Diabetic glomerulosclerosis					
Frozen-dried	2	6	Crimson	Gray-violet	Orange-brown
Zenker's	2	6	Red-violet	Orange-brown	Orange
Malignant nephrosclerosis					
Frozen-dried	1	4	Red-violet	Violet	Gray-tan‡
Zenker's	2	5	Red-violet	Red-violet	Gray-brown‡
Arteriosclerosis and pyelonephritis					
Frozen-dried	2	6	Red-violet	Gray-violet	Tan-brown
Glomerulonephritis					
Zenker's	2	5	Violet	Violet	Olive-green
Amyloidosis					
Frozen-dried*	2	5	Rose-violet	Gray-violet	Gray-yellow-tan
Zenker's	1	2	Red	Rose-gray	Tan
Lupus erythematosus					
Zenker's	2	4	Rose-purple	Violet	Olive-brown
Periarteritis nodosa					
Zenker's	1	5	Red-pink	Pink-gray	Olive-green-brown

* Included animal tissues.

† The colors described and tabulated were projected and color reproductions made with artificial light sources. Exact descriptions of such mixed colors are difficult, and for present purposes the recognition of color differences between specimens examined at the same sets of wavelengths is of greater importance than precise designation of the tints.

‡ Increased ultraviolet absorption observed.

ductions (Figs. 1 to 6) of some fields by Miss Etta Piotti, Medical Artist, Harvard Medical School, have been employed. Due to photographic technical difficulties involving color temperatures and relative film sensitivities, reproduction by transparent color photography and preparation of dye-transfer prints have so far not proved uniformly successful.

RESULTS

Normal. The normal glomerulus as observed in frozen-dried material had the following appearance (Fig. 7). First set: The nuclei of

the glomerular epithelium and endothelium were bright green with dark reticulations. This was due to the characteristic nucleoprotein absorption peak at 263 mμ common to all nuclei. Erythrocytes appeared red-pink. Glomerular stroma had a delicate red-violet shade (Table I). Second set: Nuclei and erythrocytes were similar to those in the first set. Stroma had a gray-violet color. Third set: Nuclei were light blue, erythrocytes pink. Stroma was a light gray-tan.

Zenker-fixed normal glomeruli, like all Zenker-fixed tissues studied, took on rather garish bright colors without significant alterations in the relative absorptive properties (Figs. 1, 3, and 5). Thus nuclei appeared opaque white after chromate exposure, but longer photographic exposures brought out a green color in the first and second sets and blue in the third set. Erythrocytes had a bright cerise red, with central dark non-absorptive areas resulting from leaching of hemoglobin in liquid fixative. Stromal violet was enhanced, and in the third set there was a gray-violet cast to the tan (Fig. 5).

No contribution to the problem of whether fibroblasts participate in normal glomerular structure was made. The normal stromal absorption behavior was not distinguishable from that of normal collagen in dermis, connective tissues of salivary gland, intestine, around neoplasms, and elsewhere, so far investigated.

Diabetic Glomerulosclerosis. The diabetic kidney had the most strikingly different ultraviolet absorptive behavior, compared to the normal. However, with the wave-lengths of the first set no abnormal colors were observed either with frozen-dried (Fig. 8) or Zenker-fixed material (Figs. 2 and 9).

In the second set, the Zenker-fixed stromal tissues had an orange-brown color quite unlike that of any other kidney condition (Fig. 4). In the third set, an orange color characterized the diabetic glomerular stroma whether frozen-dried or Zenker-fixed. Nuclei and erythrocytes maintained the expected normal absorptive behavior (Figs. 6, 8, and 9).

It was of interest to find that the color characteristics of diabetes apparently applied to all glomeruli, independent of the degree of their involvement in atrophy, sclerosis, or intercapillary glomerulosclerosis. Even histologically normal Zenker-fixed glomeruli had the orange color in the second and third sets and this allowed identification of a kidney from a diabetic patient with greater certainty and ease than by conventional methods. One wonders whether this would be maintained as a common property in juvenile, cortisone-induced, experimental alloxan, and other glycosuric hyperglycemic states.

Nephrosclerosis. In arteriosclerosis, particularly that exaggerated by hypertension of more malignant type, ultraviolet absorptions differed from normal in the third sets (Fig. 10). This involved a greater ultraviolet opacity at shorter wave-lengths which gave a light gray to mottled gray-tan color in the third set. Longer photographic exposures penetrated sufficiently to bring out colors identical with normal absorptions.

This increased glomerular stromal density in malignant nephrosclerosis mirrored a much more severe arteriolar densification, to be described in detail in a separate study of the ultraviolet microscopy of some vascular diseases.

In benign nephrosclerosis devoid of clinical hypertension, the glomerular ultraviolet absorptions were in the normal range. Pyelonephritis associated with glomerular atrophy and sclerosis, but without hypertension, likewise showed no unusual ultraviolet absorptive behavior.

Amyloidosis. Renal amyloidosis involving glomeruli had dull color-translated absorptions in the three wave-length sets, with violet in frozen-dried and rose-gray in Zenker-fixed specimens as the predominant colors, becoming flat gray-tan in the third sets. Only Zenker-fixed human tissue was available (Fig. 11), but frozen-dried hamster kidney from animals with severe amyloidosis complicating generalized tuberculosis[7] showed comparable ultraviolet properties.

Glomerulonephritis. In Zenker-fixed material, chronic glomerulonephritis was characterized chiefly by a bright olive-green color of the stroma in the third set. Sufficient satisfactory material was not investigated to be certain that this was a general property of the glomeruli from the various sub-types of glomerulonephritis.

Lupus erythematosus and periarteritis nodosa had third set absorptions of olive brown appearance closely similar to those of glomerulonephritis (Fig. 12). This suggested that these diseases are more closely inter-related than the other glomerular diseases studied. In one case a kidney with sarcoidosis had a rather similar ultraviolet behavior, but material was insufficient for any definite conclusions.

Discussion

The explanation of the curious specificity of ultraviolet absorption found in different glomerular diseases is not immediately apparent. The development of color-translating ultraviolet microscopy is so recent that fundamental studies and biologic findings for comparison are lacking. It was certain, however, that the observations did not depend

upon such factors as (a) thickness of section, (b) type of fixation, (c) peculiarities of individual cases or portions of tissue. The flexibility of the Polaroid microscope allowed for fractional or multiplied photographic exposures, and by their use one could become satisfied that while section thickness made tissue absorptions greater and thus colors paler or even white, longer exposures would bring out the color inherent in the absorptive properties of the tissue. Section thickness did not, for example, change green to yellow, orange, violet, blue, pink, or red. Increased density did account for the variations from normal in absorptions of glomeruli in hypertensive nephrosclerosis, but absorptions of nuclei and erythrocytes served as a guide that section thickness was not responsible for the increased absorption.

Chemical fixatives, particularly Zenker's but also formalin and formalin-Bouin, changed the ultraviolet absorption in the direction of harder, brighter, stronger colors. But the relative hues or color values were not altered. Absorptions of frozen-dried tissues were translated into soft pastel colors, like those of water-color paintings. Zenker-fixed specimens of comparable tissues showed as bright hard colors, resembling aniline dyed materials. Chromation was believed responsible for the heightening of color with Zenker's fixation.

Sufficient different fields have been examined from various blocks of kidney and from different persons to exclude individual tissues or localized tissue peculiarities of patients as causing the differences observed, with the exceptions noted. The present study has, however, been only exploratory and is an attempt to establish rather than to exhaust a field of inquiry.

An explanation of the findings, based on the present knowledge of connective tissue chemistry, may be offered. Stromal tissues, including glomeruli in general, are believed to be composed of oriented proteins, such as those in collagen fibers, surrounded by mucoprotein or glycoprotein ground substances. Loofbourow, Gould, and Sizer,[8] in attempting to separate collagen from its ground substance, found considerable changes in the ultraviolet absorptions of the successively purer collagen, particularly in the lower wave-lengths 235 to 248 mμ, where the more striking variations also were observed in the present study.

It may be suggested that glomerular stroma as it was altered by various influences came to have protein components with certain degrees of denaturation, mixed with certain proportions of mucoprotein partly depolymerized or altered otherwise to definite degrees. Each recognizable entity in glomerular disease came to have a certain proportion of chemically altered protein matrix and altered ground sub-

stance, hence providing certain ultraviolet absorption properties characteristic for the particular disease. Perhaps it was because of this chemical uniformity, such as was demonstrated by the ultraviolet absorption behavior, that each glomerular disease came to have a morphologic individuality which could be recognized and diagnosed pathologically.

Summary

Ultraviolet microscopic absorption properties of normal and abnormal glomeruli, prepared by freezing-drying or fixed in Zenker's solution, have been investigated with the Polaroid color-translating instrument. Rather strikingly different, at times specific and diagnostic, ultraviolet absorptions differing from normal were found in human diabetic glomerulosclerosis, hypertensive nephrosclerosis, amyloidosis, glomerulonephritis, and other glomerular diseases. A tentative histochemical explanation is offered, based upon the ultraviolet absorption properties of mixtures of altered protein and depolymerized mucoprotein believed to compose the abnormal glomerular stroma.

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[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Water-color reproduction of a color-translated ultraviolet microscopic field of a normal human glomerulus, Zenker-fixed, unstained. The capillaries contain cerise red erythrocytes, appearing as rings due to leaching out of centrally located hemoglobin. Opaque gray-green objects represent nuclei of glomerular epithelium and capillary endothelium, with central leaching. One opaque white chromated nucleus is present. Stroma is red-violet. Set 1 (280, 263, 240 mμ). #136626. × 950.

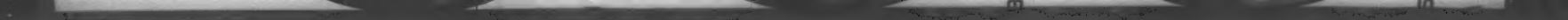
FIG. 2. Reproduction of a color-translated microscopic field of a nodule of diabetic intercapillary glomerulosclerosis, Zenker-fixed, unstained. Chromated nuclei appear white. The stroma is red-violet. Set 1 (280, 263, 240 mμ). #136449. × 950.

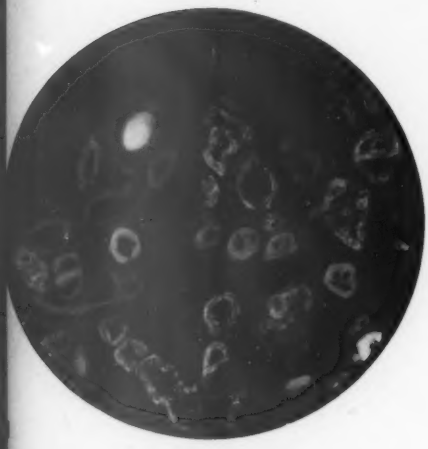
FIG. 3. The same field as shown in Figure 1, demonstrating set 2 (280, 263, 248 mμ). The major differences are the bluer appearances of nuclei, and the pinker violet color of stroma. × 950.

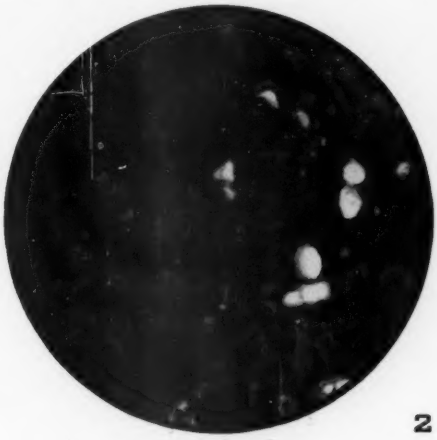
FIG. 4. The same field as seen in Figure 2, showing set 2 (280, 263, 248 mμ). The stroma has a strong orange-brown color, characteristic of glomeruli in diabetes mellitus. × 950.

FIG. 5. The same field as shown in Figures 1 and 3, showing set 3 (248, 240, 235 mμ). Erythrocytes and stroma appear gray-violet-tan. Nuclei are faintly blue. × 950.

FIG. 6. The same field as seen in Figures 2 and 4, demonstrating set 3 (248, 240, 235 mμ). The stroma has an orange color, similar to that in Figure 4. × 950.







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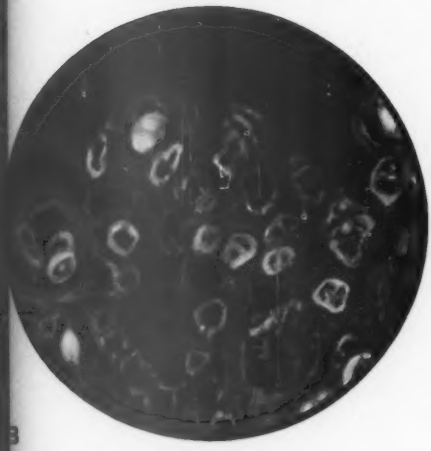

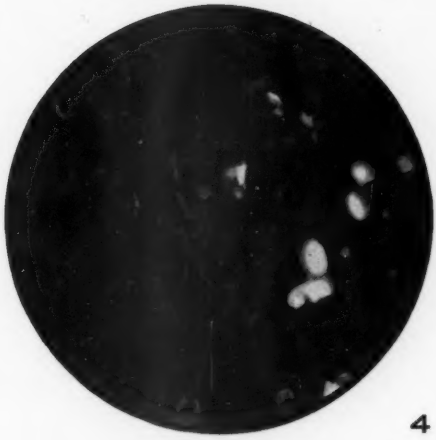

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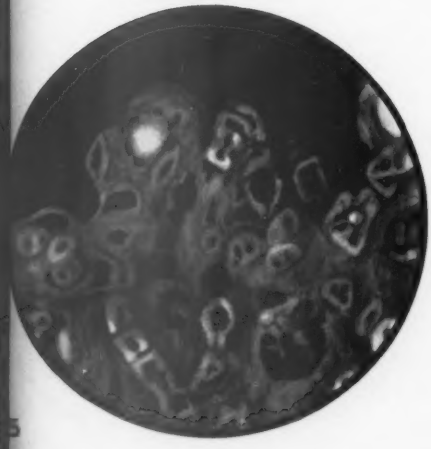

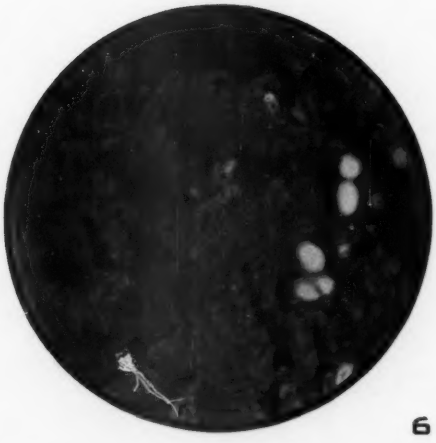

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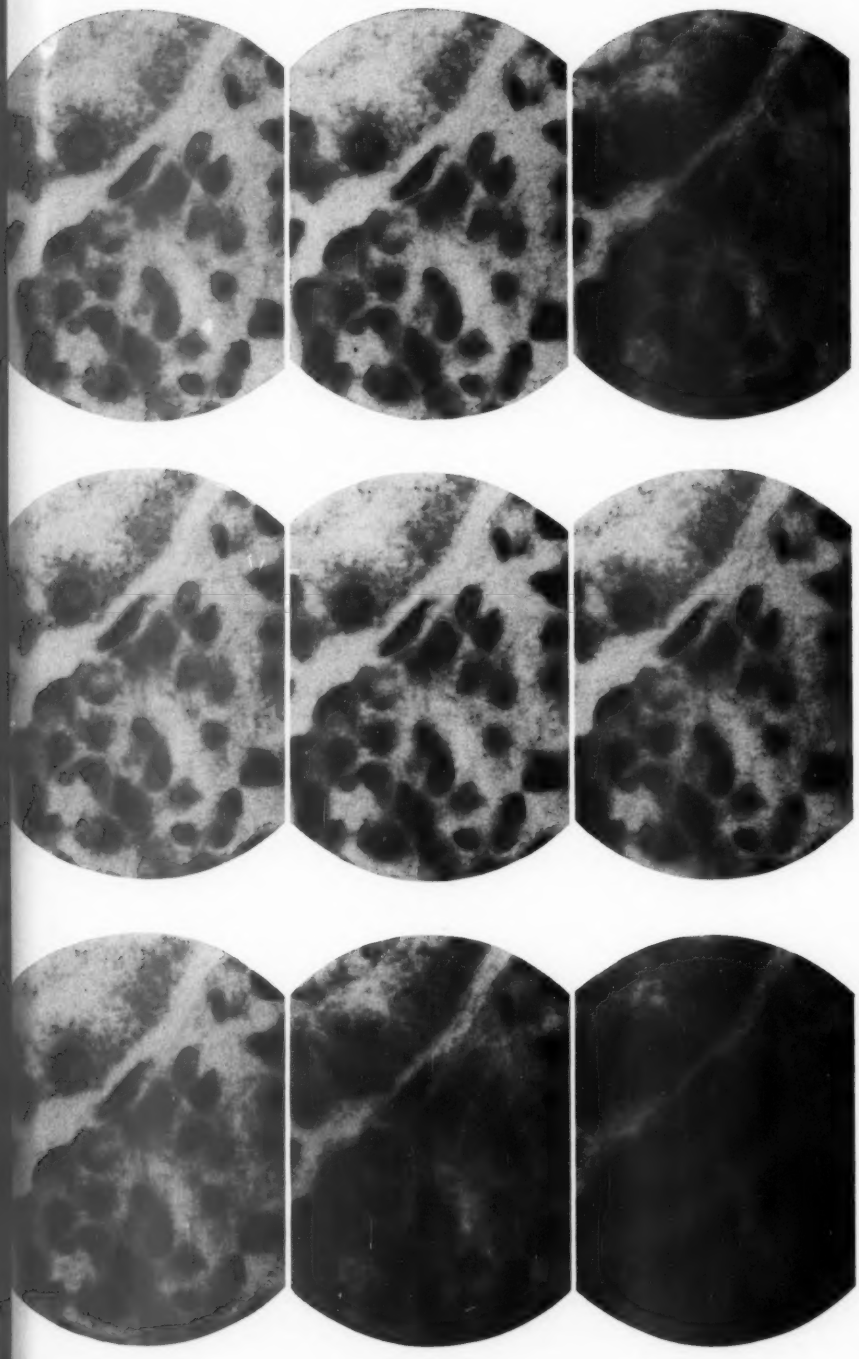
Figures 7 to 12 are all arranged in the manner explained for Figure 7. These are positive photographic prints, so that the darker shades represent increased color intensities. The left-hand vertical row was translated into blue, the middle vertical row into green, and the right-hand vertical row into red. Resulting colors of cells and stroma are given in the text and in Table I. Nuclei generally appeared green in set 1 due to the nucleoprotein absorption peak near 263 mμ. × 700.

FIG. 7. Reproductions of portions of original 35 mm. film strips, showing part of a frozen-dried normal human glomerulus, examined by color-translating ultraviolet microscopy. A section of kidney tubule is present at the upper left. The upper horizontal row forms the first set: From left to right, photomicrographs are at 280, 263, and 240 mμ. The middle row is the second set: From left to right, photomicrographs are at 280, 263, and 248 mμ. The lower row is the third set: From left to right, photomicrographs are at 248, 240, and 235 mμ. #140677. × 700.

WOLVERINE

X



7

FIG. 8. Reproductions of portions of film strips, arranged as for Figure 7. A frozen-dried glomerulus from a human diabetic patient is demonstrated. Color-translated ultraviolet absorptions were notably abnormal in set 3, shown as the lower horizontal row, which gave a dark orange-brown color. #140494. × 700.

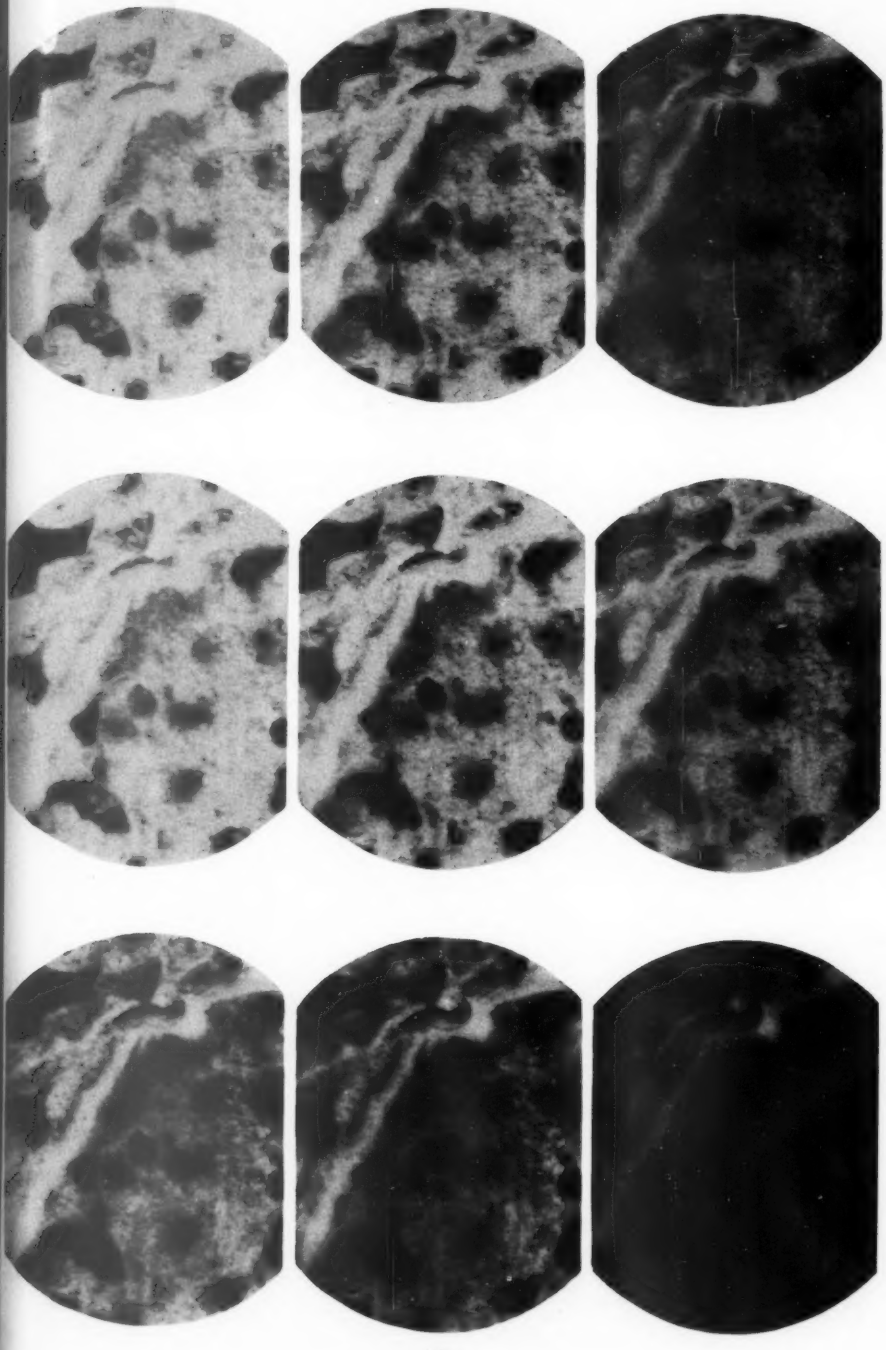


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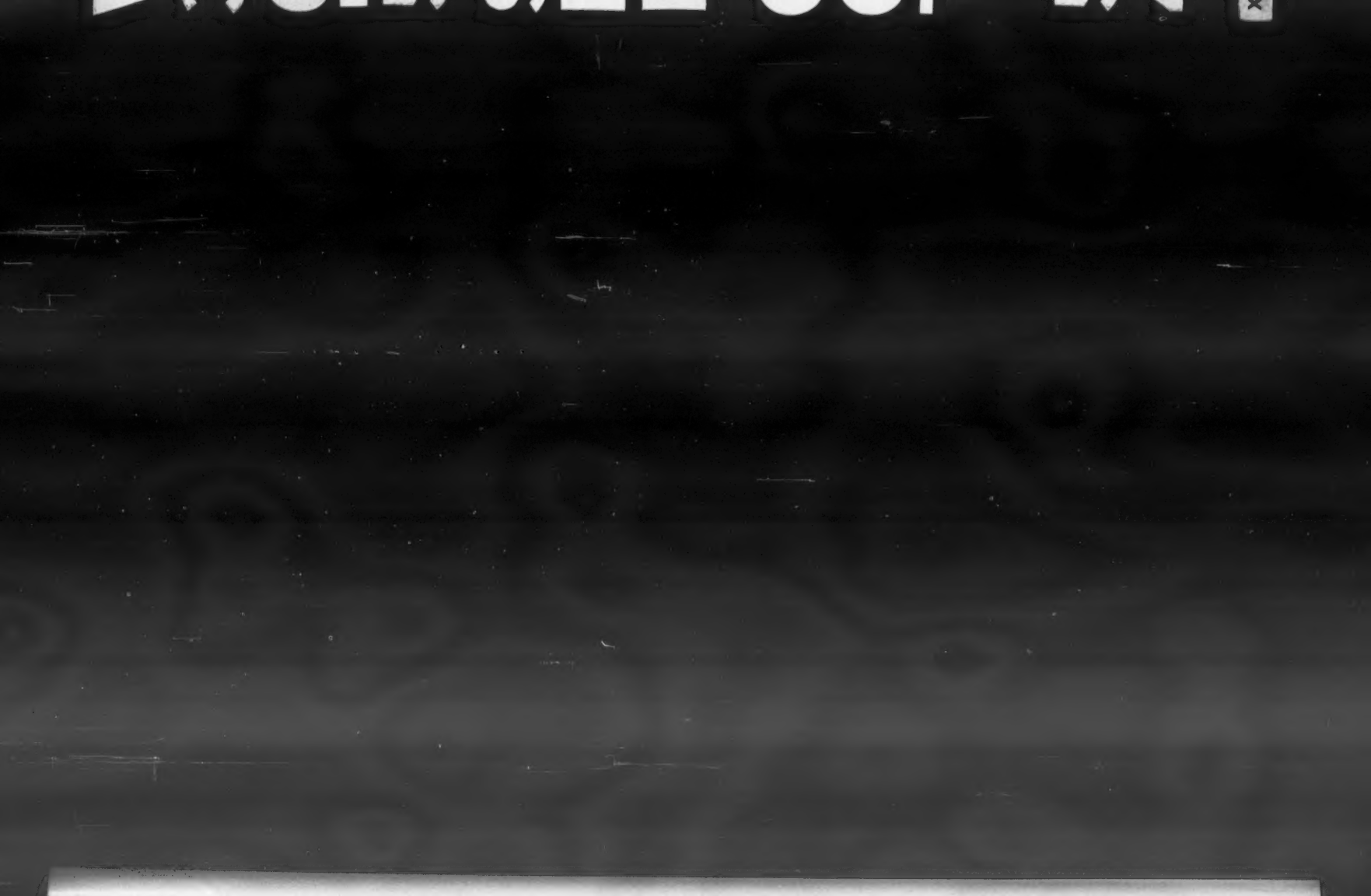
FIG. 9. Reproductions from film strip of a Zenker-fixed human glomerulus with nodular intercapillary sclerosis. The color-translated stromal absorptions were the same as illustrated in Figures 2, 4, and 6 from another portion of the same patient's kidneys. #136449. × 700.



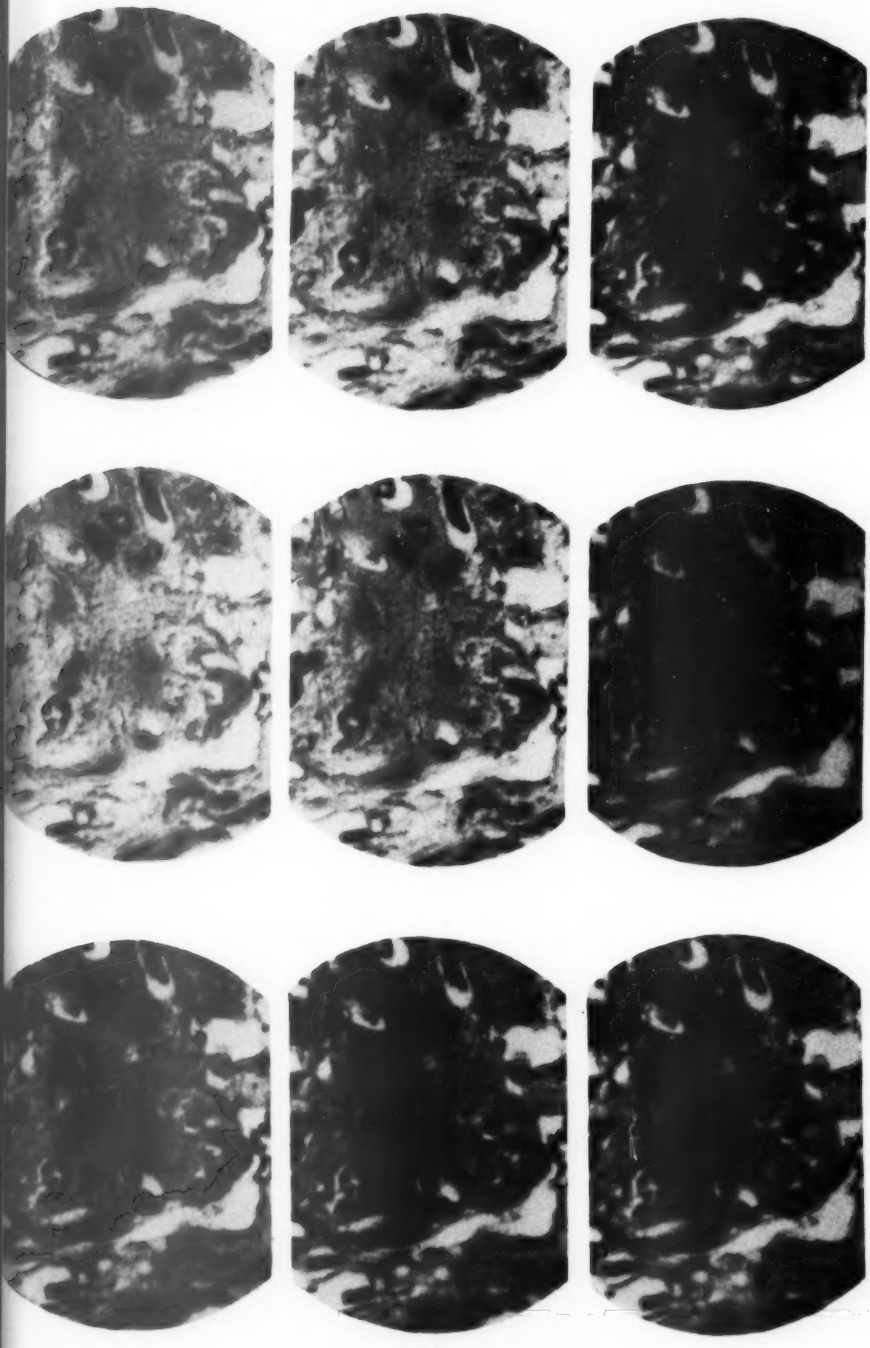


FIG. 10. A similar reproduction of part of a Zenker-fixed human glomerulus, from a case of severe arteriolar nephrosclerosis. The most striking abnormality with ultraviolet photomicrography was the increased density of stroma, shown as darker shades of gray in the positive prints illustrated. #132800. × 700.

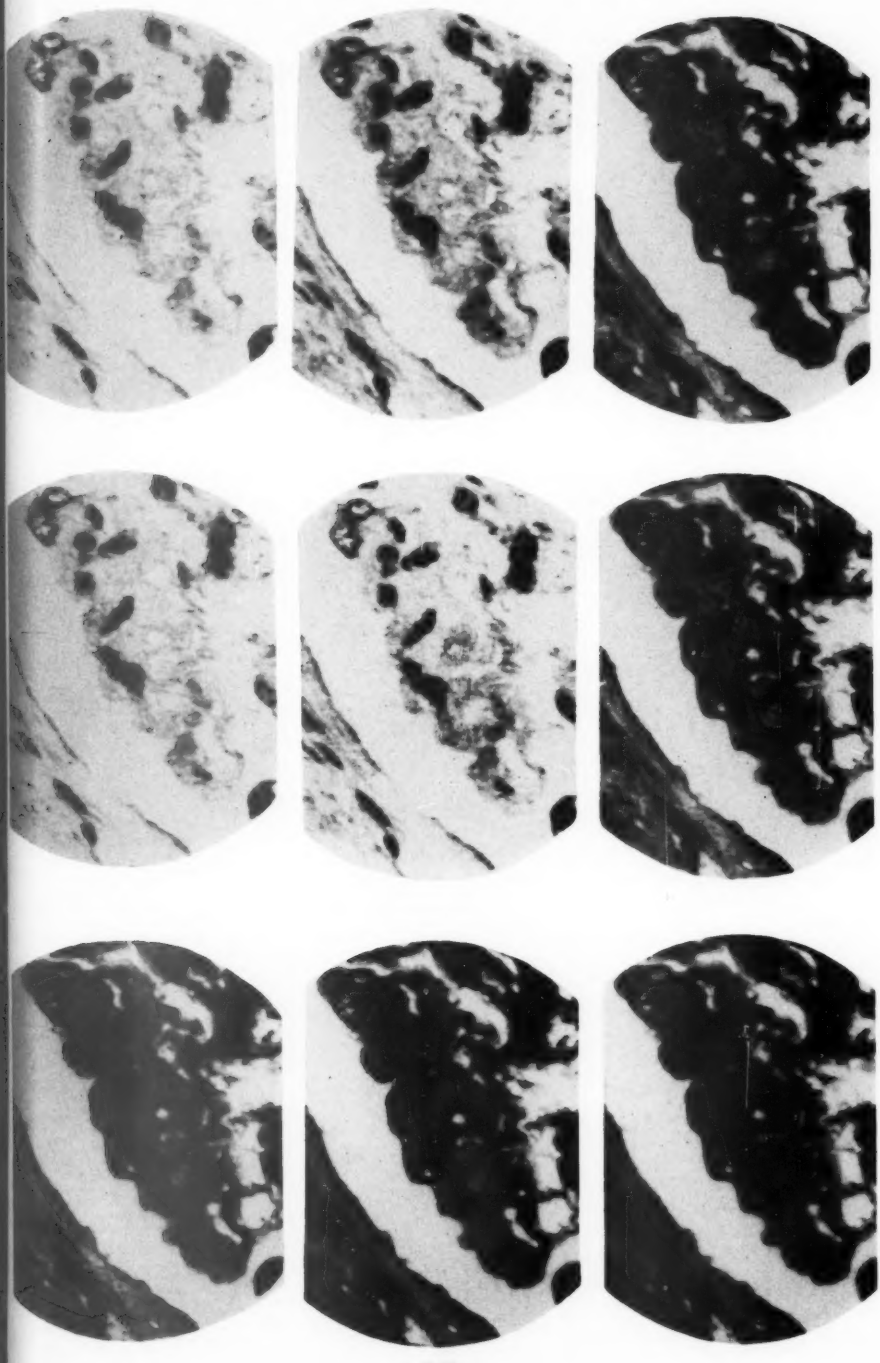


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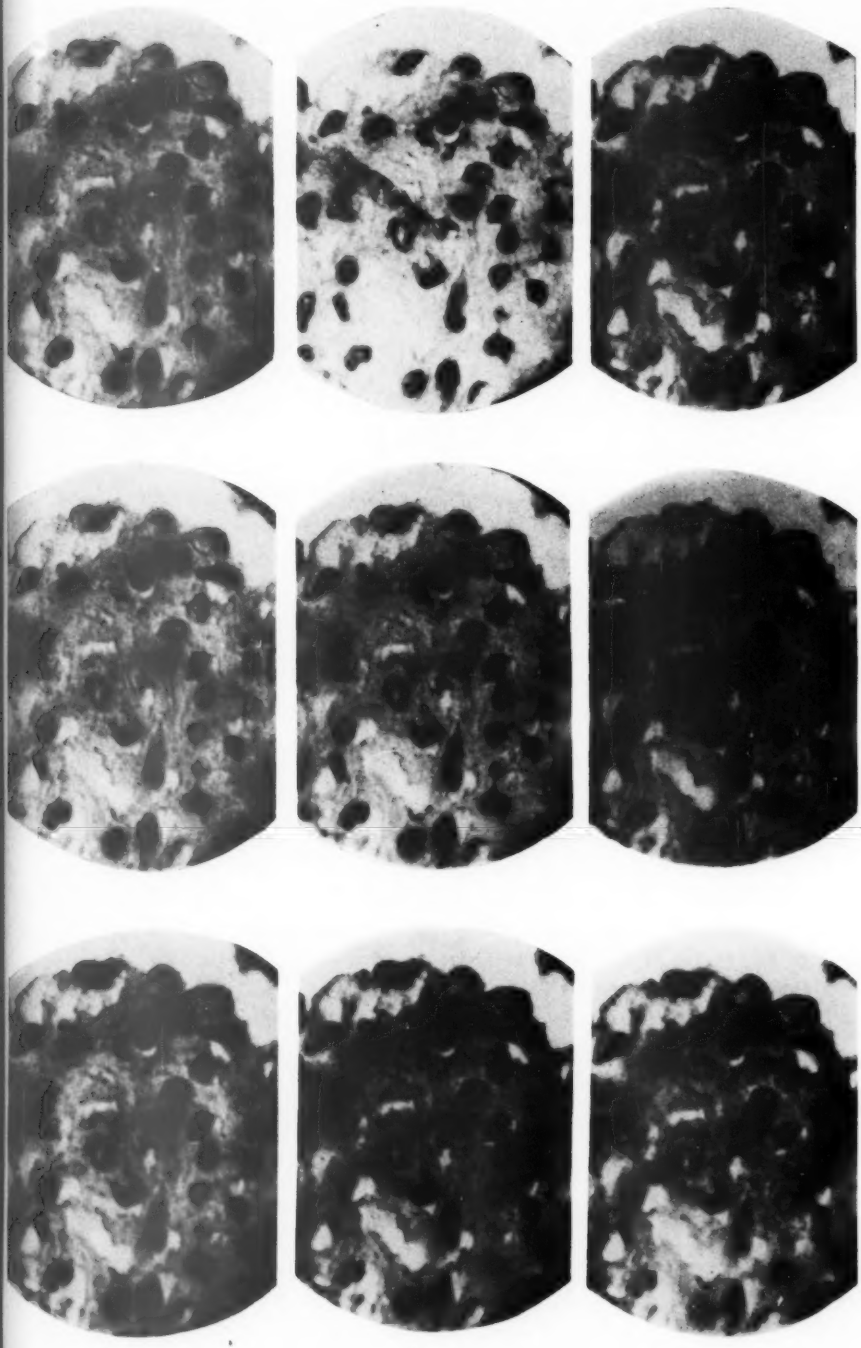
FIG. 11. A portion of a human glomerulus with amyloid deposit, Zenker-fixed. The gray-appearing nodules of amyloid had rose-gray and tan appearances in the second and third sets. #100845. × 700.

FIG. 12. Part of a Zenker-fixed glomerulus from a case of disseminated lupus erythematosus. The olive-brown appearance of stroma in the third set resembled stromal absorptions observed in glomeruli from cases of glomerulonephritis and periarteritis nodosa. #142925. × 700.



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THE UPTAKE OF COLLOIDAL THORIUM DIOXIDE BY THE ARTERIAL LESIONS OF CHOLESTEROL ATHEROSCLEROSIS IN THE RABBIT

Its Significance in Relation to Pathogenesis *

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Although other theories have been proposed and supported by various authors,[1] it has been generally accepted for many years that the nutrition of the inner layers of arterial walls is provided by fluid derived from the plasma, which permeates the intima from the lumen and seeps through the arterial wall to be drained off by the venules and lymphatics of the outer layers. While this is a perfectly logical idea, confirmatory experimental evidence is virtually confined to observations on the permeation of colloidal dyes through the intima from the lumen of the aorta.[2] As a corollary to this concept it has been assumed also that the lipids that accumulate in the intima in atherosclerosis are derived from the nutritive fluid that filters through it from the lumen. Indeed, this is one fundamental assumption on which is based the imbibition or infiltration theory of the pathogenesis of atherosclerosis as propounded by Ribbert,[3] Aschoff,[4] Anitschkow,[5] and others. However, the form in which lipids enter the intima, their mode of transit through the lining endothelium, and the manner of their progressive aggregation in the subendothelial layer of the intima are problems yet to be elucidated.

It appeared to us that additional evidence bearing on these questions might be accumulated by the use of identifiable colloidal materials other than dyes and by the study of their entry into preformed experimental atherosclerotic lesions, as well as their behavior when introduced into the circulation of animals with normal arteries. Some years ago, Plewes[6] observed the presence of thorium dioxide in the foam cells of an aortic atherosclerotic plaque at necropsy in a patient in whom thorotrast had been used for diagnostic purposes. This suggested the possibility that thorotrast might be a suitable agent for such experimental studies. These studies have now been in progress for several years and some of our observations have been reported briefly elsewhere.[7]

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Materials and Methods

In an initial experiment, 15 young adult rabbits were fed for 2 to 3 months on a diet containing corn oil and cholesterol, the latter in a dose of 0.6 to 0.8 gm. daily. This procedure led to the development of hypercholesterolemia and moderately severe atherosclerosis of the aorta. At the end of the period of cholesterol feeding, colloidal thorium dioxide in the form of warmed thorotrast* was injected intravenously into each rabbit in a dose of 2 to 3 cc. per kg. of body weight. The animals were killed at intervals of from 5 minutes to 4 days after the injection of thorotrast. Six rabbits that had not been fed cholesterol were injected with colloidal thorium dioxide in the same manner and were killed at times ranging from 30 minutes to 12 hours following injection. Careful necropsy examinations were carried out on all animals and the organs and many cross sections of the aorta were subjected to microscopic examination in paraffin sections and in frozen sections stained for fat.

Forty-six young adult white rabbits were employed in a second experiment. Five of these animals were not fed cholesterol but were injected intravenously one, two, or three times with thorotrast in the same dose as before at intervals of 24 hours; and were killed 24 hours after the last injection. The remaining 41 rabbits were fed 1 gm. of cholesterol mixed with 92 gm. of rabbit chow and 7 gm. of corn oil daily for 5 days each week. Samples of serum taken at the beginning and at the end of each experiment were analyzed for their content of cholesterol, lipid phosphorus, and fatty acids of neutral fat. Twenty-four hours before the animals were to be killed each was injected intravenously with 3 cc. of warm thorotrast per kg. of body weight. Blood specimens, taken at random times following the injection of thorotrast, were collected from the ear opposite that into which the material was injected and were allowed to clot. The clots were then fixed in formol-saline solution, embedded in paraffin, sectioned, stained, and examined microscopically for the presence of thorium dioxide. The animals were killed in small groups at weekly intervals after from 1 to 8 weeks of cholesterol feeding. Their organs and multiple blocks of aorta were examined microscopically in paraffin sections and in frozen sections stained for fat.

Observations

With one exception, the aortic intimas of the total of 11 control animals that had not been fed cholesterol did not contain microscopically visible amounts of thorium dioxide. The exception occurred in a

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patch of thickened, fibrotic intima that covered an area of spontaneous calcific sclerosis in the media of the aorta. In this location only, the lining endothelial cells were seen to contain fine granules of thorium dioxide. The endothelial cells were somewhat swollen but otherwise were of normal appearance. The endothelial cells covering another similar lesion in the aorta of another animal, however, showed no thorium in the plane of the microscopic section.

In the first experiment, the 15 cholesterol-fed rabbits showed prominent atherosclerotic lesions of the aorta at necropsy. Some of these contained particles of thorium dioxide. No thorium was seen in the aortas of those animals killed within 10 minutes following the injection of thorotrast, but it was found in trace amounts in the atherosclerotic lesions of some of the rabbits killed 20 minutes after injection. In animals killed 3 hours after injection the substance could be seen as rather coarsely aggregated granules, while in those animals killed 24 hours after receiving thorotrast the particles of thorium dioxide were often densely clumped in those cells in which it was present. Thorium was not found in the aortic atherosclerotic lesions of every animal killed from 20 minutes to 3 hours after injection but it was invariably present in animals killed 12 hours or longer after injection. It was present in areas where atherosclerotic lesions occurred but was absent from the intervening areas that were not involved by atherosclerosis. It was found in both the lining endothelial cells and in the foam cells of the atherosclerotic lesions.

The thorium dioxide as observed in the histologic sections had the characteristic bronze, metallic appearance and agglomerated, particulate form that has been described by other authors.[8,9] In the lining endothelial cells the granules lay in the cytoplasm and were usually minute (Figs. 1 and 2). The endothelial cells containing thorium were always more swollen than their neighbors and were often ovoid. Endothelial cells containing thorium, sometimes in rather large amounts, were often numerous and prominent over the atherosclerotic lesions. Much larger amounts of thorium eventually accumulated in the globular foam cells that composed the bulk of the atherosclerotic lesions (Figs. 1, 2, and 3), but its earliest appearance in these cells was in the form of minute granules scattered through the cytoplasm. With the passage of succeeding hours the aggregates of thorium became progressively coarser and more numerous. Thorium was also found in small amounts in some of the fibroblastic cells in atherosclerotic lesions that contained them. Granules of thorium were not observed in the intercellular ground substance except in a few microscopic preparations and then in circumstances in which it was impossible to be sure that

the granules had not been artificially displaced from neighboring cells.

The distribution of thorium among the cells of the atherosclerotic plaques was generally irregular. It was observed to occur in single, isolated, lining endothelial cells, in small groups of them or in many adjacent endothelial cells always in relation to atherosclerotic lesions. Sometimes the subjacent foam cells or fibroblasts contained thorium and sometimes they did not. Aggregates of thorium dioxide were seen in lipid-filled foam cells beneath endothelial cells that were free of the substance (Fig. 3) and the reverse was also true. Single foam cells and groups of adjacent foam cells were observed to contain the material while other cells between them contained none (Figs. 2 and 3). In some early lesions all of the foam cells in a single section were completely filled (Fig. 1).

In spite of the general irregularity of distribution, it was noted that granules of thorium dioxide were accumulated first and most abundantly in the layers of foam cells closest to the endothelial surface rather than in those closest to the media (Figs. 2 and 3). In sections of the aortas of 4 animals the thorium dioxide was found to have accumulated most abundantly at the periphery of many of the atherosclerotic plaques, both in endothelial cells and in foam cells rather than at random over their surfaces (Fig. 4). For several animals longitudinal sections of the aorta were made after the proximal end had been marked for identification by injecting India ink into the adventitia. In no case was it apparent that the proximal part of an atherosclerotic plaque had accumulated significantly more or less thorium dioxide than the distal part. The distribution of thorium appeared to be fairly uniform around the whole periphery of such plaques and was absent only in the depths of their central areas.

Mitotic figures similar to those described and illustrated in a previous publication[10] were seen in the foam cells, both in cells that contained thorium and in cells that did not. In addition, one endothelial cell that contained a few minute particles of thorium was found with a mitotic figure in anaphase (Fig. 5). No instance of mitosis in an endothelial cell was encountered in our previous study of mitotic activity in the lesions of experimental cholesterol atherosclerosis.

No thorium was found in the smaller arteries of other organs and tissues except in the coronary arteries of one animal in which two small atherosclerotic plaques showed the presence of thorium granules in endothelial and foam cells in a distribution similar to that observed in the aortic lesions. The distribution of thorium dioxide in the additional organs examined was similar to that described by other

authors.[8,9] In those animals that had been fed cholesterol, thorium was found in abundance in the lipid-filled reticulo-endothelial cells of the liver, spleen, and other organs.

In the second experiment, there were 17 animals that failed to show any evidence of aortic atherosclerosis either on gross examination or in the sections studied microscopically. The majority of these animals had been fed cholesterol for periods no longer than 3 weeks. Nevertheless, in 4 of these rabbits that had been fed cholesterol for 1 week and in which the level of serum cholesterol had risen to three to five times the normal value, small granules of thorium dioxide were found in several slightly swollen aortic endothelial cells. In one animal fed cholesterol for 3 weeks the total cholesterol content of the serum reached 1240 mg. per cent. The aorta presented a normal intima in microscopic sections except that particles of thorium were found in a number of swollen endothelial cells. The same was true of one other rabbit fed cholesterol for 8 weeks, during which time the total serum cholesterol rose to only 280 mg. per cent. The remaining 11 rabbits in this group exhibited neither aortic atherosclerosis nor particles of thorium dioxide in the intima of the aorta. Five of these animals were moderately hypercholesterolemic but 6 were only mildly hypercholesterolemic or showed normal levels of cholesterol in their sera when they were killed.

Six rabbits that were fed cholesterol for 3 to 8 weeks showed only very slight microscopic changes in the aorta consisting of localized areas of swelling of endothelial cells and of the subendothelial layer of the intima without histologically demonstrable fat. Thorium dioxide in minute granules was present in some of these swollen endothelial cells. The remaining 18 rabbits presented microscopic or gross atherosclerotic lesions of the aorta. In early microscopic lesions in which subendothelial foam cells contained easily demonstrable lipids, granules of thorium dioxide were present in the overlying endothelial cells and in the foam cells. More advanced atherosclerotic plaques presented the appearances detailed in preceding paragraphs.

One hundred and six samples of peripheral blood, taken from 30 minutes to 24 hours after the injection of thorotrast, were examined in paraffin sections of clot. All but two showed granules of thorium dioxide. In various samples the substance was found free in the plasma or contained in polymorphonuclear leukocytes and in monocytes. Various combinations of these extracellular and intracellular situations were observed. Accumulations of thorium dioxide in polymorphonuclear leukocytes never amounted to more than a few granules scattered

in the cytoplasm, but in monocytes the numbers of granules ranged from a few to numbers so large as to fill the cytoplasm and to distend the cell to a large size. These large cells showed no vacuolation of their cytoplasm or any other evidence of lipid content. Monocytes filled to capacity with thorium granules were rare in specimens collected from 30 minutes to 8 hours after the injection of thorotrast. In blood samples obtained from 18 to 24 hours after injection, monocytes well filled with thorium granules were far more numerous.

Discussion

The results of these experiments show a consistent uptake of intravenously injected colloidal thorium dioxide in atherosclerotic lesions by the foam cells lying in the layer nearest the lumen. These cells accumulated thorium earlier than the deeper ones and always in larger quantities. Indeed, in advanced lesions the foam cells nearest to the media contained no thorium at all and the deposit was confined to the foam cells nearer the lining endothelium. These observations clearly imply entry of the colloidal thorium dioxide from the arterial lumen through the lining endothelial membrane and not from the vasa vasorum. This in turn substantiates the theory that other colloidal substances, including those of lipid character, enter the intima from the lumen through the endothelium and accumulate in atherosclerotic lesions. While the observations in these experiments do not demonstrate the passage of thorium dioxide from the lumen into the normal parts of the intima, neither do they deny the possibility of such an occurrence. It may be merely that the substance passes through the normal intima without accumulating there in visible amounts. Nevertheless, the permeation of colloidal thorium from the lumen into established atherosclerotic plaques is entirely in harmony with the concept of normal nutrition of the intima by infiltration of nutritive fluid from the lumen.

It is of interest that many of the foam cells of the atherosclerotic lesions, though already well filled with lipids, avidly accumulated particles of thorium dioxide in great numbers and in a very short time. This suggests a remarkable degree of functional activity on their part. There is no reason to doubt that they could increase their accumulations of lipids just as rapidly as they took up thorium if lipid substances were presented to them at the same rate and in suitable form. In view of this potential acquisitive activity, it is not apparent why individual foam cells, unlike their neighbors equidistant from the endothelium, failed to take up any thorium, unless it is assumed that their

position was by chance such as to exclude thorium dioxide from contact with them or else that the functional potential of the foam cells varies from time to time.

The idea has been proposed by Leary[11] and elaborated by Gordon[12] that the foam cells of atherosclerotic lesions have their origin as cells of the reticulo-endothelial system in the spleen, liver, lungs, or elsewhere which become loaded with lipids *in situ* and later migrate by way of the blood stream to invade the arterial intima by penetrating the endothelial lining. According to this hypothesis, the lipid content of the atherosclerotic lesions is brought in from a distance by this kind of intracellular transport. Our observations indicate that this is not true of thorium dioxide. Thorium granules accumulated in large numbers in the lipid-filled reticulo-endothelial cells of the cholesterol-fed rabbit in the spleen, liver, and elsewhere, but the thorium-containing cells found in the circulating blood were free from cytoplasmic vacuolation or any other sign of the presence of lipid. In this respect they differed completely from the reticulo-endothelial cells of the liver and spleen and also from the foam cells of the atherosclerotic lesions, all of which contained thorium granules and abundant lipids. No cells were found in the circulating blood that could account for the intracellular transport of thorium from the reticulo-endothelial system to the aortic intima during the relatively brief period when large amounts of thorium were being accumulated in the aortic lesions.

To the extent that an analogy can be drawn between the behavior of lipids in the blood stream and of intravenously injected colloidal thorium dioxide, it is apparent that our observations are in conflict with Leary's[11] hypothesis. More direct evidence that this theory is not valid is provided by the experiments of Simonton and Gofman[13] and of Harris,[14] who labelled the reticulo-endothelial cells of rabbits with identifiable materials before and during cholesterol feeding. Ultimate examination of the atherosclerotic lesions that developed during the experiments showed that no significant migration of the labelled reticulo-endothelial cells into the lesions had occurred, though the lesions contained abundant lipid-filled foam cells.

The accumulation of thorium in the lining endothelial cells overlying atherosclerotic lesions was an entirely unexpected finding. Thorium did not accumulate in the normal aortic endothelium of control rabbits injected with thorotrast. This is in conformity with the observations of Efskind[15] who found that thorium dioxide did not appear in the lining endothelium of the arteries of normal rabbits after intravenous injection of thorotrast. In our experiments the aortic endothe-

lium of cholesterol-fed rabbits also was free of the substance except in swollen endothelial cells that lay in most instances in relation to obvious atherosclerotic lesions. It seems apparent, therefore, that the ability of lining endothelial cells to take up and aggregate colloidal thorium dioxide into microscopically visible particles is an acquired characteristic that develops during the course of cholesterol feeding. Its development is regularly accompanied by some degree of swelling of the endothelial cells in question.

In one control animal not fed cholesterol, swollen endothelial cells lying over a spontaneous calcific lesion of the media also exhibited the capacity to accumulate thorium. Accordingly, it is impossible to regard this manifestation of altered function on the part of the endothelial cells as a specific effect of cholesterol feeding. Indeed, this observation suggests the possibility that the lining endothelial cells may undergo the observed morphologic and functional changes during cholesterol feeding simply because of the accumulation of lipid deposits beneath them which displaces the endothelial cells lumenward. This process might be thought of as providing a mechanical or chemical stimulus that causes alteration of cellular form and function.

While this seems a perfectly plausible explanation of the relation between the morphologic and functional alterations of lining endothelial cells and the intimal or medial lesions that lay beneath them, it is equally possible that the localization of the lipid deposits in the arteries of the cholesterol-fed rabbits may have been determined by the prior occurrence of functional changes in the lining endothelium in certain localized areas. Although our experimental observations do not demonstrate such a property, it is quite conceivable that swelling of endothelial cells and their acquisition of the ability to aggregate colloidal thorium dioxide may be accompanied by an increase in their permeability. This not only might be expected to favor the entrance of lipids into the intima in the affected areas but might even be a *sine qua non* to the process of lipid deposition. It appeared highly important, therefore, to determine if possible whether the acquired changes in the lining endothelial cells of the aorta preceded or followed the first appearance of lipid deposits in the arteries of cholesterol-fed rabbits.

It was in the hope of determining this point that our second experiment was carried out in which the rabbits were fed cholesterol for brief periods, given an intravenous injection of thorotrast, killed 24 hours later, and their aortas subjected to microscopic examination. In some of these animals fed cholesterol for periods as short as 6 days, granules of thorium dioxide were found in small groups of swollen

endothelial cells that were encountered in otherwise normal areas of the intima which lacked any trace of lipid deposit. This observation gives clear proof of the rapid acquisition of altered properties on the part of the endothelial cells within a very short period of cholesterol feeding. However, it is impossible to be sure that lipid deposits, though not demonstrable in the sections examined, did not exist in close proximity but outside the plane of the section. Wherever the smallest definite lipid deposit was found in the intima, the overlying endothelial cells had already acquired a slightly swollen appearance and the ability to take up and aggregate the intravenously injected colloidal thorium dioxide. Nevertheless, the use of cross sections of the aorta that were not serial sections rendered it impossible to conclude with certainty that the described morphologic and functional alterations of the lining endothelial cells preceded the first appearance of lipid deposits in the intima.

We are convinced that the conventional method of examining histologic cross sections of arteries lacks sufficient precision for the microscopic study of the very earliest changes of atherosclerosis such as those with which we are dealing here. This conviction is fully confirmed by preliminary observations already made, employing a method that we have devised and described elsewhere[16] which permits the microscopic examination of large areas of the arterial intima from its surface. By this method the topographic relationship of endothelial cells containing granules of thorium dioxide to deposits of lipids in the underlying intima can be readily demonstrated in a single preparation. Further studies employing this technique may settle some of the questions to which our present observations do not provide final answers.

It seems remarkable that lining endothelial cells of the arteries in cholesterol-fed rabbits should take up and accumulate colloidal thorium dioxide injected into the circulation when they show no tendency to accumulate lipid materials in appreciable amounts. Of course, the two kinds of substances are quite different in chemical constitution and very different in physical state as well. The lining endothelium must be, or must become during cholesterol feeding, permeable to both, since both must pass through the endothelial membrane to accumulate in the foam cells that lie beneath. It is all the more remarkable, therefore, that some of the thorium should be arrested and aggregated in the endothelial cells while quantities of lipid materials pass through the membrane leaving scarcely a trace in the lining endothelium. This fact is a fortunate one, however, for it has permitted in these experiments a clear demonstration (for the first time, so far as we are aware)

that a localized change in the biologic properties of lining endothelial cells develops in close association with the first appearance of lipid deposits in the intima of the aorta in cholesterol-fed rabbits.

Since thorotrast has been employed as a radiologic contrast medium, it seemed worth while to determine whether the quantities of thorium dioxide that accumulated in the experimental atherosclerotic lesions were sufficient to throw the lesions into contrast in roentgenograms. Though the quantities of thorium present in some of the lesions appeared under the microscope to be considerable, we were unable to demonstrate corresponding shadows in roentgenograms when aortas containing well developed lesions infiltrated with thorium dioxide were opened and laid flat on the photographic plate during a suitable exposure to x-ray beams of appropriate quality.

Summary and Conclusions

Colloidal thorium dioxide in large quantities was injected intravenously into normal rabbits and into rabbits that had been prepared by the previous feeding of cholesterol for from 1 week to 3 months. The animals were killed 5 minutes to 4 days following the injection of thorium dioxide. The aortas and other tissues were subjected to a searching microscopic examination for the presence of the characteristic granular aggregates of thorium dioxide.

It was observed that thorium dioxide did not accumulate in the normal arterial endothelium and intima of normal rabbits nor in the endothelium or intima of normal appearance that lay between atherosclerotic lesions in the aortas of cholesterol-fed rabbits. However, thorium dioxide accumulated rapidly and consistently in some of the endothelial cells and in superficially situated foam cells that constituted the principal cells of both small and large atherosclerotic lesions.

The observations clearly imply the entry of the colloidal thorium dioxide from the arterial lumen through the lining endothelial membrane and not from the vasa vasorum. The permeation of the substance into established atherosclerotic lesions is consistent with the concept of normal nutrition of the intima by perfusion of fluid from the vascular lumen and substantiates the theory that other colloidal substances, including those of lipid character, enter the intima from the lumen through the endothelium to accumulate in atherosclerotic lesions.

To the extent that lipids in the blood and intravenously injected colloidal thorium dioxide may be assumed to behave similarly, the observations deny the hypothesis that the foam cells in atherosclerotic

plaques have their origin as lipid-filled reticulo-endothelial cells in the spleen, liver, and lungs that subsequently migrate in the blood stream with their contained lipid to invade the intima (Leary's hypothesis).

The acquisition by endothelial cells of the ability to accumulate thorium dioxide during cholesterol feeding appeared to be a non-specific effect possibly due to some mechanical or chemical stimulus or to a local alteration in cellular and intimal permeability. An attempt was made in short-term cholesterol feeding experiments to determine whether lining endothelial cells of the rabbit's aorta acquired the ability to take up and aggregate colloidal thorium dioxide prior to or after the first appearance of lipid deposits in the intima. This question could not be resolved, but it was shown that this change in the biologic properties of lining endothelial cells occurred at a time at least as early as the earliest appearance of lipid deposits in the subendothelial layer of the intima as seen in histologic cross sections of the aorta stained for fat. The possible significance of these observations on the altered behavior of the arterial endothelium in relation to the pathogenesis of experimental cholesterol atherosclerosis in the rabbit is discussed.

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LEGENDS FOR FIGURES

FIG. 1. A small atherosclerotic lesion of the aorta. The swollen endothelial cells on its surface contain small, dark granules of thorium dioxide. Fine and coarse granular aggregates of the same material are accumulated in the cytoplasm of the lipid-filled foam cells that constitute the lesion. A clear halo can be seen around some of the granules. Zenker-formol fixation. Hematein-phloxine-saffron stain. × 900.

FIG. 2. A large atherosclerotic lesion of the aorta containing several layers of cells. The endothelial cells are somewhat swollen and small granules of thorium dioxide are seen in some of them. The foam cells immediately subjacent to the endothelium contain much thorium dioxide while those that lie deeper and closer to the media contain little or none. Zenker-formol fixation. Hematein-phloxine-saffron stain. × 900.

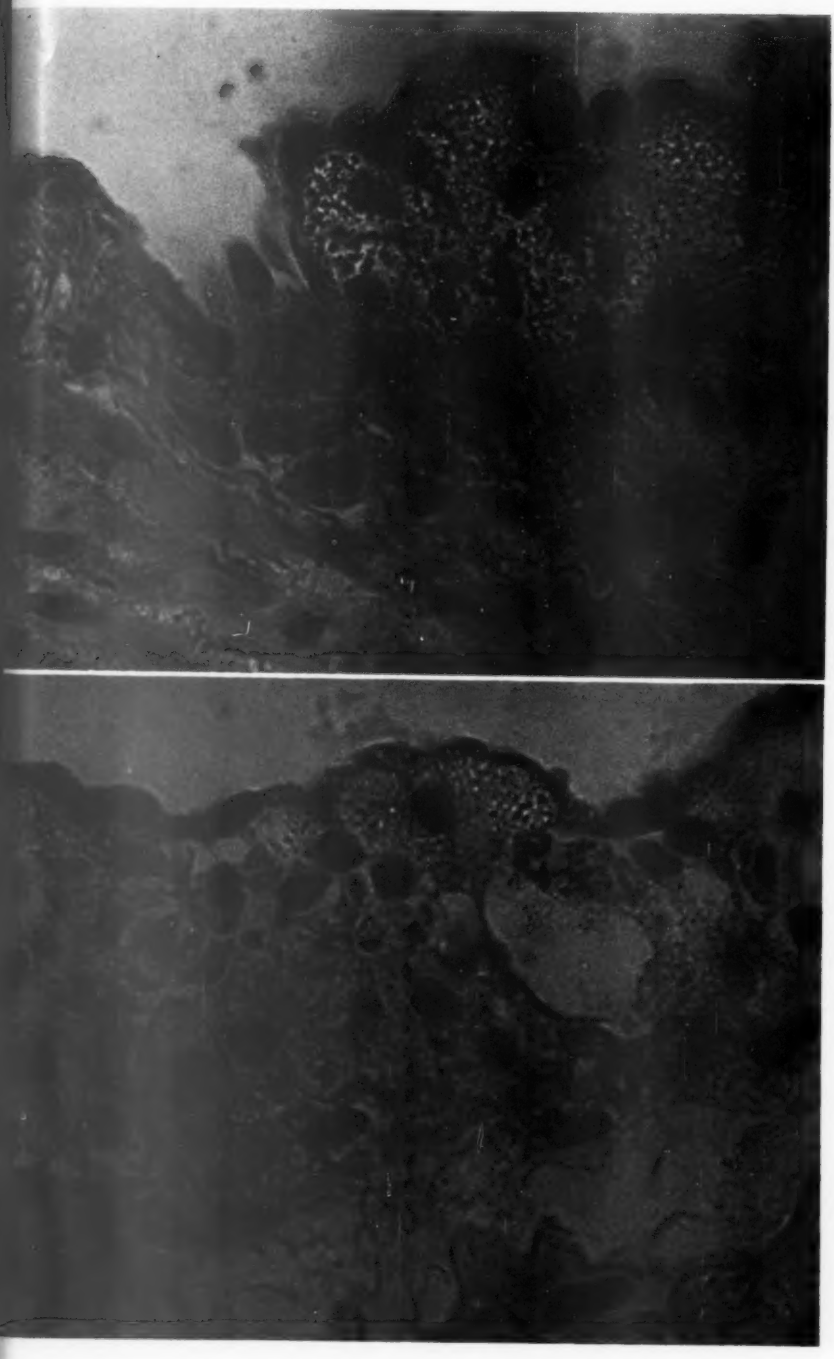

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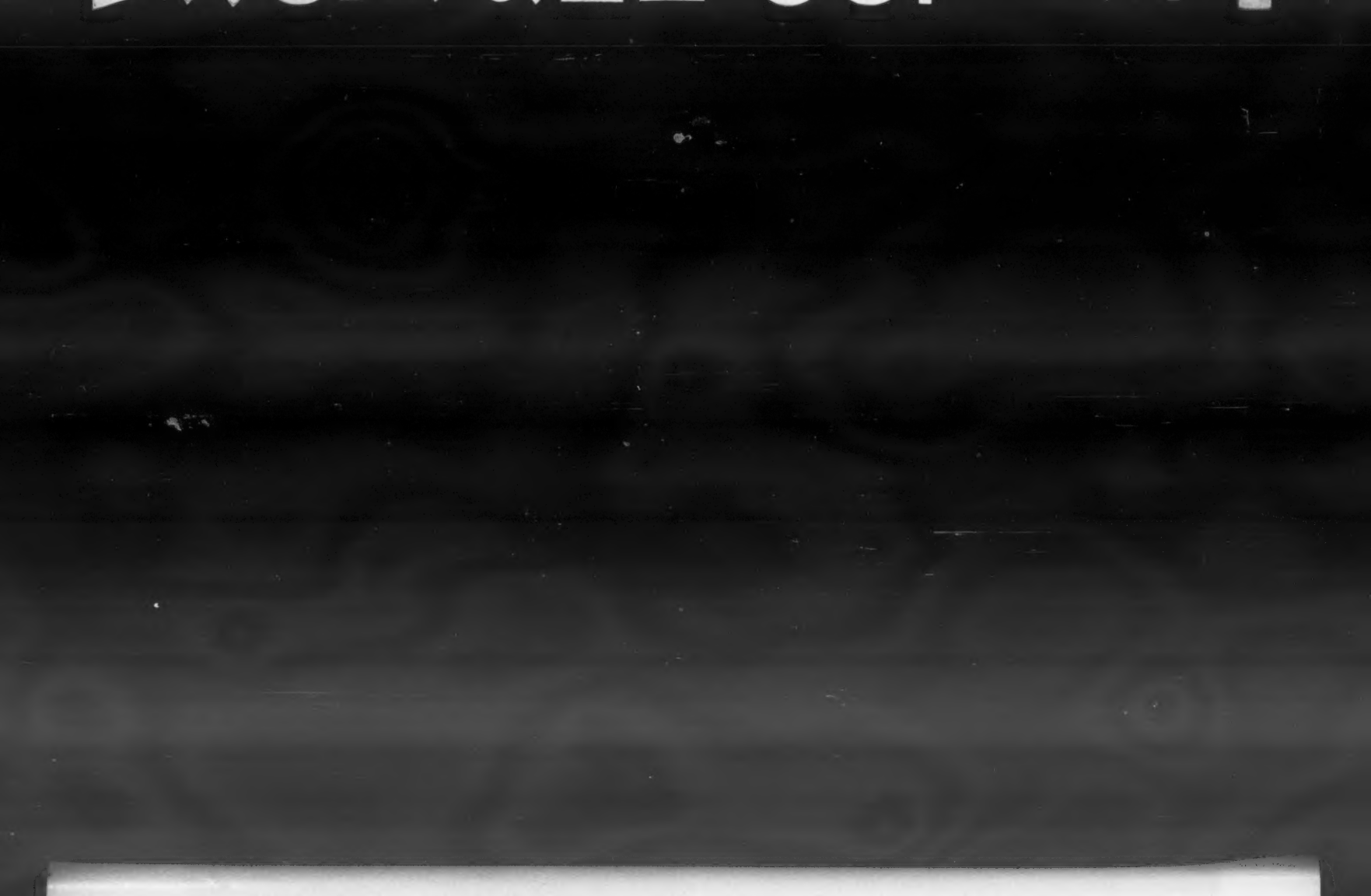
FIG. 3. A large atherosclerotic lesion of the aorta. Thorium dioxide granules are not visible in the endothelial cells and the latter are not swollen. Some of the subjacent foam cells contain granules of thorium while immediately neighboring cells do not. The deeper layers of cells are free. Zenker-formol fixation. Hematein-phloxine-saffron stain. × 900.

FIG. 4. Photomicrograph illustrating the peripheral margin or shoulder of an atherosclerotic lesion of the aorta. The lesion is several cell layers in thickness toward the left hand side but only one cell thick on the right. This thinner portion is covered by a partly detached film of clotted blood. The subjacent endothelial cells are swollen and contain granules of thorium. The underlying foam cells contain much thorium dioxide that is visible even at this relatively low magnification. The cells that constitute the thicker and more central part of the lesion contain only small amounts of the material or none at all. Zenker-formol fixation. Hematein-phloxine-saffron stain. × 210.

FIG. 5. Photomicrograph illustrating an endothelial cell in mitotic division. The division is in anaphase. The cell cytoplasm contains granules of thorium. To the right, immediately under the endothelium, is a foam cell containing much thorium dioxide. Formol-saline fixation. Hematoxylin and eosin stain. × 1100.



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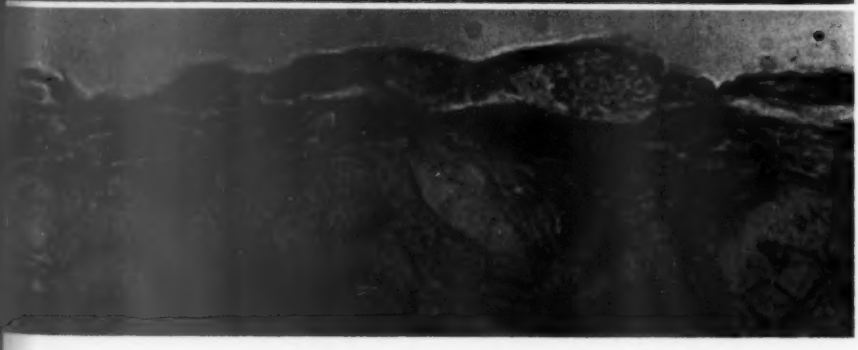

5

BLASTOMATOUS OLIGODENDROGLIA AS SATELLITES OF NERVE CELLS

A Study with Silver Carbonate *

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The knowledge of oligodendroglia was greatly advanced by Del Río Hortega,[1-3] who investigated these cells with the silver carbonate and silver chromate techniques. By these methods he established a close relationship of glia of this type to the nerve fibers and described satellite systems in the white matter of the brain and in the sympathetic and spinal ganglia, which are built predominantly of oligodendroglia. With new variants of this technique Del Río Hortega[4] was able to impregnate the elements of oligodendroglioma, and with the same procedures it is also possible to investigate blastomatous satellite structures of this neoplasm, which is the objective of the present investigation. In the normal tissue Del Río Hortega described five types of oligodendroglial cells: (1) small, round elements with a central nucleus and several delicate processes which coil around the nerve fibers (type 1, Robertson) (Fig. 1A); (2) larger cells of similar appearance, but with T-shaped processes which form a distinct "perimyelinic" network (type 2, Cajal) (Figs. 1B and C); (3) large elements with oval bodies which are located near the nerve bundle; their processes, shaped as spirals, surround the neuraxis with a complicated fibrillar network (type 3, Paladino) (Figs. 1D and E); (4) fully differentiated oligodendroglia or elements of Schwann (Figs. 1F). The cells of Schwann are tubular structures which surround the nerve fibers with a network "in depth" and are built of rings, "funnels," and fibers (Cajal,[5] Nemiloff,[6] Del Río Hortega[1]). (5) In the sympathetic and spinal ganglia Del Río Hortega[2,3] discovered a new type of oligodendroglia which he called spirocyte (Fig. 2). All of the cells form an important part of the supporting system of the neurons and the nerve fibers, which, according to Del Río Hortega,[2,3] consists of two morphologically different but closely linked components: (1) the perisomatic glia, which surround the body of the neuron with a network of cells and fibers, and (2) the periaxonic glia which support the axons. There are some morphologic differences between the basically similar satellite systems of the peripheral and the central nervous systems. In the former the perisomatic network is built exclusively of one variety of

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perisomatic gliocytes (Del Río Hortega[2,3]); in the latter, of astroglia, oligoglia, and microglia (Del Río Hortega,[1] De Castro[7]). The periaxonic component of the satellite system of the sympathetic and spinal ganglia is built of spirocytes and elements of Schwann, and in the gray centers of the four types of oligodendroglia peculiar to the central nervous system.

Blastomatous oligodendroglia form a supporting system of their own, which closely resembles that of their normal counterpart. In the blastomatous satellite structures there can be distinguished also perisomatic and periaxonic parts, and the tumor cells imitate normal oligodendroglia and their relationship to the neuraxis. For proper evaluation of the illustrations it must be borne in mind that the morphology of the oligodendroglioma in silver carbonate preparations is characterized by a polymorphism which rivals that of glioblastoma. The blastomatous elements imitate all types of oligodendroglia, including elements of Schwann, blastomatous astrocytes, and astroblasts (Bailey and Bucy,[8] Del Río Hortega[4]), and form numerous anaplastic types. All of these elements are capable of acting as satellites to the neurons and nerve fibers. Therefore, it is not surprising that satellite systems built of neoplastic cells show great morphologic variations. In spite of this there can be distinguished several types which are predominant: (1) satellite systems which consist of perisomatic elements only; (2) systems built of periaxonic cells; (3) transitional types; (4) well developed systems with distinctly separate perisomatic and periaxonic components.

The perisomatic network built of perisomatic gliocytes in various states of development is demonstrated by Figure 3: in the upper part of the illustration a damaged neuron is surrounded by processes of several blastomatous elements: that on the right (A) is a transition between an oligodendrocyte and an astrocyte; the cell on the left and those above and below the neuron are differentiated as oligodendrocytes (B, C, D). In the left lower corner, a multipolar, elongated, anaplastic oligodendroblast partly surrounds the neuron; at the lower right, neuron and satellite are in an advanced state of disintegration. There is no periaxonic part. A different type of perisomatic network is shown in Figure 4, in which a large perisomatic element with Y-shaped processes, distinctly differentiated as an oligodendrocyte of type 2, surrounds the neuron with a dense fibrillary network; the periaxonic component is not present. Perisomatic structures with elements differentiated as oligodendrocytes of type 1 are common (Fig. 5); the processes of these cells form a rudimentary periaxonic structure; the satellite at A is an oligodendroblast, that at B is differentiated as

an oligodendrocyte. There are numerous systems built of elements resembling oligodendroglia of type 3. These cells have a large, round nucleus and one long, well developed process which runs parallel to the neuron (Fig. 6A). Another cell, similar in type, is seen at the opposite side of the neuron; both cells form a definite perisomatic, and a rudimentary periaxonic, structure which is reinforced by thick, clumsy, and disorganized processes of an anaplastic oligodendroblast (Fig. 6B). Satellite systems of this type frequently tend to develop rudimentary periaxonic structures built of dense, intertwined fibers. In these satellite structures blastomatous cells of periaxonic type act simultaneously as perisomatic and periaxonic satellites (Fig. 7). Among the blastomatous elements there frequently occurs a cell characterized by a large, round, or oval nucleus, and one long, thick, plasmatic process. These elements resemble oligodendroglia of type 4 (equivalent of Schwann elements) (Fig. 8). As periaxonic satellites they surround the axons with homogeneous plasmatic bodies and partly with a dense network of spirals; a poorly developed perisomatic network may be present (Fig. 9). There are also numerous transitional types in which perisomatic components consist of a few poorly differentiated cells with numerous long processes which surround the axon with a continuous chain of spirals and coils. These cells act simultaneously as perisomatic and periaxonic elements (Fig. 10). Occasional neoplastic satellite systems may attain a high degree of differentiation of both components. In these structures the perisomatic network is built of cells differentiated as oligodendrocytes of type 1, and the periaxonic part of homogeneous plasmatic bodies typical of the elements of Schwann (Fig. 11). Both components of the system remain within the limits of their respective provinces and are distinctly different. These structures closely imitate their normal counterparts.

Comment

As has been stated, in normal tissue the neurons are surrounded by a system of satellite cells in which two histologically different components can be distinguished: (1) a perisomatic capsule which surrounds the body of the neuron and is built of oligodendrocytes, astrocytes, and microglia, and (2) a perisomatic component built of elements of Schwann (oligodendroglia of type 4) which surround the nerve fibers with a continuous plasmatic tube. It cannot be doubted that these systems are capable of nutritive and "trophic" functions (Del Río Hortega[1]), that they are indispensable for the normal functioning of the neurons, and therefore must be regarded as satellites of the latter.

The blastomatous oligodendroglia are able to form satellite systems

of their own which so closely resemble their normal counterparts that they can be analyzed without difficulty. In the blastomatous structures perisomatic and periaxonic components can be easily recognized, but there are many pathologic types. The perisomatic component is usually better developed and can be easily identified; it is built of blastomatous oligodendrocytes, oligodendroblasts, and anaplastic elements, and frequently presents a close imitation of the corresponding normal structure. The periaxonic part is frequently not present or remains in a rudimentary state. This latter structure is built for the greater part of elements resembling oligodendroglia of types 3 or 4, which surround the axons with a plasmatic tube or numerous coils resembling elements of Schwann.

The various blastomatous elements which compose these structures, such as oligodendrocytes, oligodendroblasts, and cells which resemble elements of Schwann, frequently do not respect their anatomical provinces as do normal cells, but act simultaneously as perisomatic and periaxonic satellites. This accounts for the occurrence of transitional types, among which four sufficiently differentiated varieties could be distinguished, in some of which both component parts were highly developed.

The processes of blastomatous oligodendroglial cells, including the anaplastic types, form spirals and coils which are also characteristic of normal oligodendroglia and enable them to maintain an intimate relationship with the neurons. This suggests that the blastomatous elements are able to carry out some nutritive functions and to support the life of the neurons for a certain period of time.

Although blastomatous satellite systems are common in the oligodendrogliomas, they do not involve extensive areas but remain restricted to small foci in the gray matter. The manner in which they replace normal cells could not be determined by the technical procedures at our disposal.

Technical Notes

Fixation

For the silver impregnation of normal and pathologic glia and of tumors of the glioma group, preservation in brom-formalin is requisite.

Formalin (*neutral*); 37 or 38 %	15.0 cc.
Distilled water	85.0 cc.
Ammonium bromide	2.0 gm.

Biopsy material is preferably fixed immediately after removal (in operating room). Duration of fixation is determined by the size of the tissue block: 16, 24, or 48 hours. Fixation of the unsectioned brain is a poor substitute. The brain is cut into slices, 0.5 cm. thick, and preserved in large quantities of fixing fluid. Brain tissue preserved in acid formalin or with other organs is useless.

The tissue is cut on a freezing microtome; tumor tissue not thicker than 10 μ, and, if possible, 5 μ; cerebellum 15, 20, or 25 μ. Sections are preserved in brom-

formalin in which they can be kept for several weeks. Tumor tissue can be impregnated successfully for several months, astroglia and neurons up to 3 years.

Stock Solution

Silver nitrate solution, 10% ..... 100 cc.
Sodium carbonate solution, 5% ..... 300 cc.

Shake and add (drop by drop) concentrated ammonium hydroxide until the precipitate is dissolved; *avoid excessive ammonia.* Keep in a dark brown bottle. This solution can be used for a period of about 3 months. Use only clear solutions.

The silver carbonate solution is used in three concentrations:

1. Strong concentration: stock solution ..... 100 cc.
   distilled water ..... 25 cc.
2. Medium concentration: stock solution ..... 100 cc.
   distilled water ..... 100 cc.
3. Weak concentration: stock solution ..... 100 cc.
   distilled water ..... 275 cc.

Triple Impregnation

This is the most important technique for the impregnation of the gliomas.

1. Wash sections 1 to 3 minutes (not longer) in 100 cc. of distilled water with 20 to 30 drops of concentrated ammonia.
2. Wash in distilled water (1 to 2 minutes).
3. Transfer sections into 50 cc. of a 2% silver nitrate solution with 20 drops of pyridine, for 2 hours at 45° C. Place the dish with sections on a carefully regulated hot plate (45° C.) or water bath of 60° C.
4. Transfer sections (*without washing*) into 50 cc. of weak silver carbonate with 20 drops of pyridine, for 24 to 48 hours at room temperature and protected from light.
5. Transfer sections (without washing and one at a time) into 2 per cent silver nitrate ammonia for 20 seconds. (Method of preparation follows.)
6. Reduce in 1% formaldehyde (2 to 3 minutes).
7. Wash carefully in distilled water.
8. Tone in a 0.2% gold chloride bath at room temperature for 20, 30, 40, or 60 minutes.
9. Wash in distilled water.
10. Fix in a 5% solution of sodium thiosulfate, 5 minutes.
11. Wash carefully in distilled water.
12. Place (by floating method) on a slide. Dry completely at room temperature, clear, and mount.

The silver nitrate ammonia solution is prepared by adding concentrated ammonium hydroxide (drop by drop) to 2% silver nitrate solution. Stir until the brown precipitate is dissolved, *avoid excess of ammonia.* Stronger concentrations of silver ammonia (3, 4, or 5%) may be used occasionally; the weaker solution of 2% is preferable.

Work always with large quantities of solution; 50 cc. of silver carbonate or silver nitrate are sufficient for 30 sections 1 by 1 cm. Large series of well impregnated sections can be prepared without difficulty. The intensity of impregnation should be tested at intervals of 4, 6, 12, or 24 hours in silver carbonate. Insufficiently impregnated sections can be reinforced after procedure 7, by placing them (one at a time) into weak silver carbonate for 3 to 5 seconds (not longer). Reduce in 1% formaldehyde.

One % formaldehyde is prepared by mixing 25 cc. of 10% solution with 225 cc. of distilled water; add 10 drops of pyridine to 250 cc. of 1% solution; change formalin frequently.

Astroglia Technique

This is a selective method for impregnation of astroglia.

1. Fixation in brom-formalin (requisite).
2. Wash in ammonia water.
3. Wash in distilled water.
4. Transfer sections into 50 cc. of weak silver carbonate plus 20 drops of pyridine, at 45° C. for 20, 30, or 40 minutes. Sections will turn light brown.
5. Reinforce the impregnation (2 or 3 sections at a time) in 2% silver ammonia solution for 20 seconds.
6. Without washing in water reduce in 1% formaldehyde.
7. Tone in 0.2% gold chloride 20 to 30 minutes.
8. Wash in distilled water.
9. 5% thiosulfate, 5 minutes.
10. Wash thoroughly, clear, and mount.

This is a very simple, extraordinarily reliable, and efficient procedure, far superior to the older methods. Serial sections in large numbers, uniformly impregnated, can be prepared within one hour.

For detailed information consult Scharenberg and Zeman.[9]

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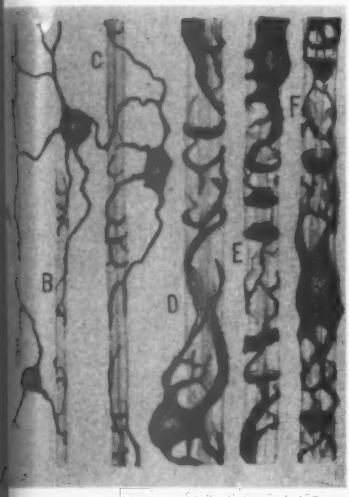
LEGENDS FOR FIGURES

FIG. 1. Oligodendroglial elements modified after Del Río Hortega; A, type 1; B-C, type 2; D-E, type 3; F, type 4 (Schwann).

FIG. 2. Spirocyte with numerous coils which surround the axon; human gasserian ganglion (Scharenberg). Double impregnation. × 1500.

FIG. 3. Elements of blastomatous oligodendroglia which form the perisomatic network of the satellite system. A, transition between a blastomatous oligodendrocyte and an astrocyte; B, C, and D are oligodendrocytes. Satellite at lower left is an anaplastic cell; at right satellite and neuron are degenerated. Triple impregnation. × 1000.

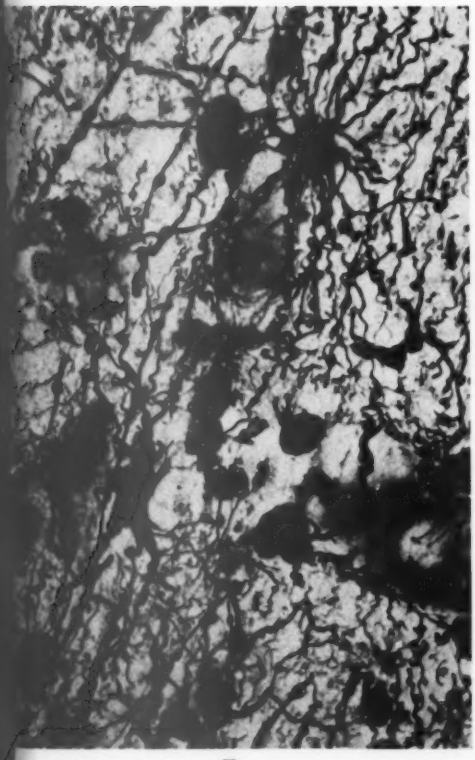
FIG. 4. Large blastomatous oligodendrocyte of type 2 with Y-shaped processes which form a dense perisomatic network. Astroglia technique. × 1000.



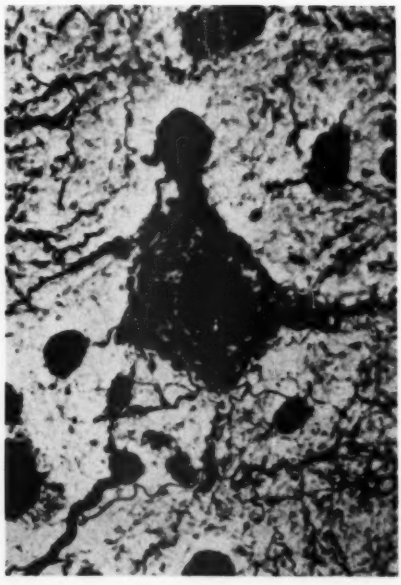
1

B

2



A



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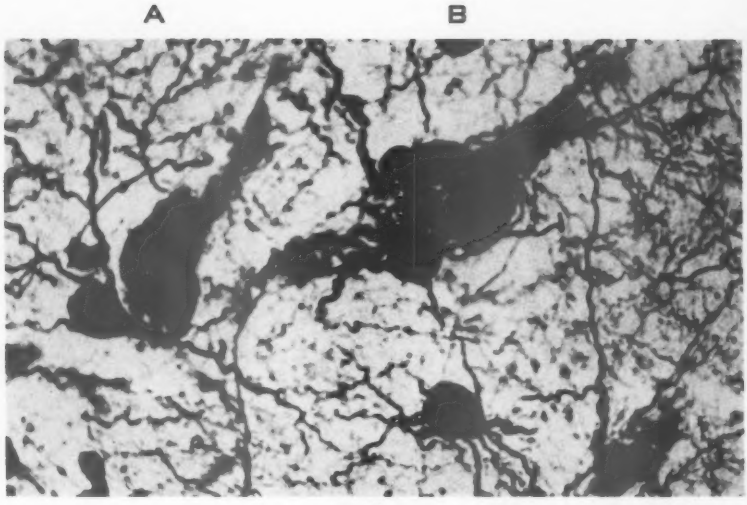
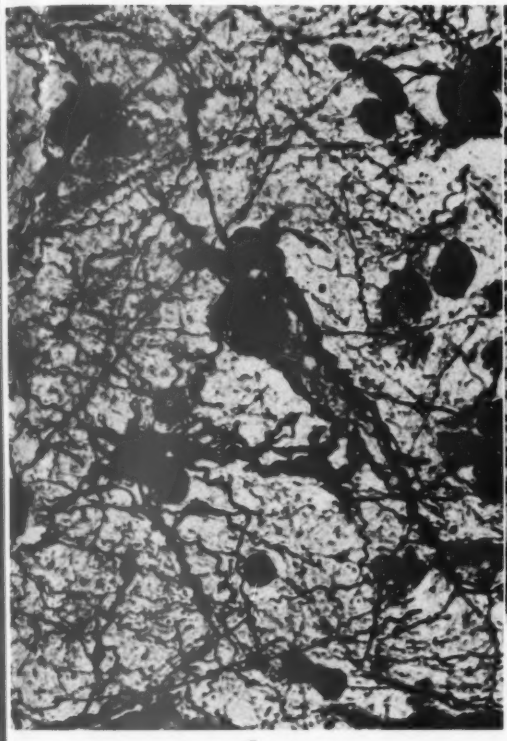


FIG. 5. Perisomatic structure built of elements differentiated as oligodendroglia of type 1; the periaxonic component is very poorly developed. Triple impregnation. × 1000.

FIG. 6. Blastomatous oligodendroglia of type 3 which forms a distinct perisomatic and rudimentary periaxonic component. Triple impregnation. × 1000.

FIG. 7. Blastomatous elements of type 3 which act simultaneously as perisomatic and periaxonic satellites. Triple impregnation. × 1000.

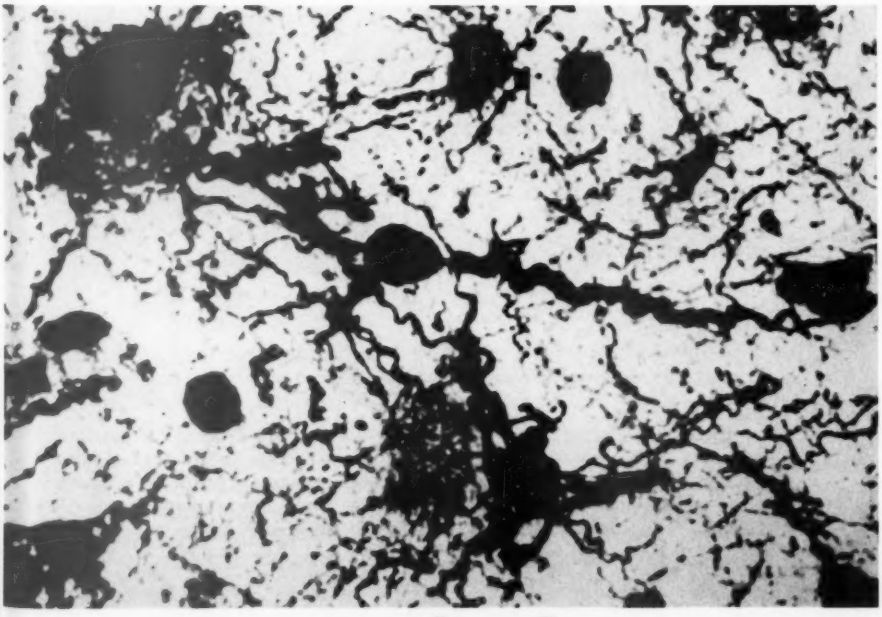
FIG. 8. Blastomatous oligodendroglia of type 4, with one long, thick, plasmatic, and several irregular processes which reinforce the perisomatic network of two neurons (A and B). Triple impregnation. × 1000. Print enlarged × 2.



6

7

A



B B

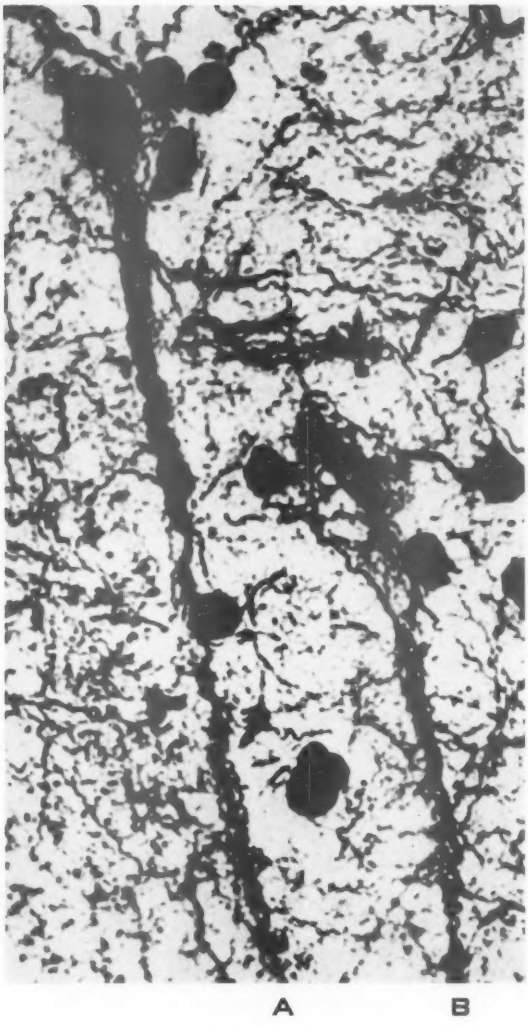


FIG. 9. Periaxonic satellite (A) of type 4 (Schwann) which surrounds the proximal segments of the axon with a homogeneous plasmatic tube and forms numerous coils around the distal part of the axon. The perisomatic structures are poorly developed. In the satellite system under (B) both components are well developed. Triple impregnation. × 1000. Print enlarged × 2.

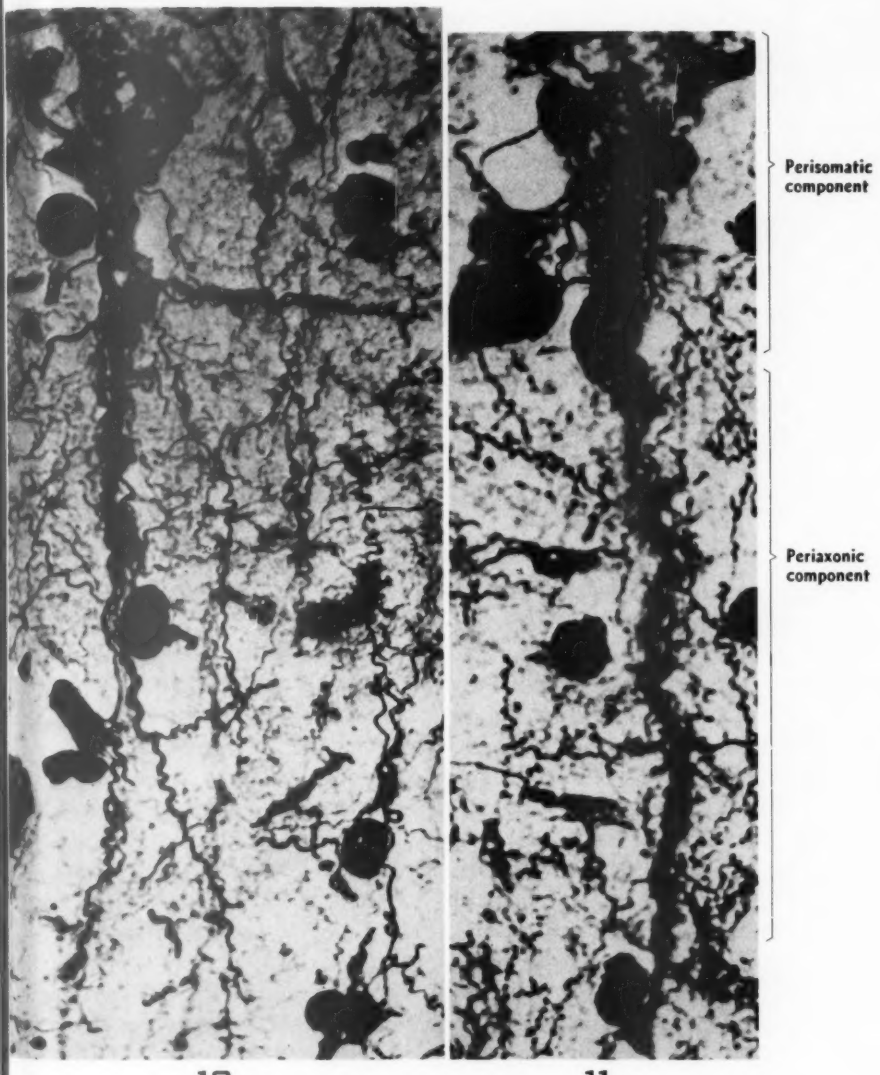


FIG. 10. Blastomatous periaxonic satellites of type 4 (Schwann) which form a well developed periaxonic component, with numerous long processes, shaped as spirals and coils. The perisomatic part is very primitive. Triple impregnation. × 1000. Print enlarged × 2.

FIG. 11. Well developed blastomatous satellite system with distinct perisomatic part, built of oligodendrocytes of type 1 and equally well developed periaxonic part, built of elements of type 4 (Schwann). Triple impregnation. × 1000. Print enlarged × 2.

THE BLOOD SUPPLY OF NEOPLASMS IN THE LIVER *

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During an investigation of the hepatic circulation in rabbits, we noted that hepatic tumors failed to stain when India ink was injected into the portal vein, whereas hepatic tissue between the tumors became intensely black.[1] However, when the hepatic artery was injected the tumors as well as the liver tissue became stained. It seemed, therefore, that the tumors were supplied mainly, perhaps exclusively, by arterial blood, in contrast to the predominantly portal supply of hepatic tissue.[2]

In the experiments to be reported, the blood supply of a variety of neoplasms growing in the liver of several species was estimated by various injection methods. Evidence is presented to show that the blood supplying all malignant neoplasms tested was largely, if not exclusively, arterial. A preliminary summary of some of these findings has been reported.[3]

MATERIALS AND METHODS

All determinations of blood supply were based on the results of injection of colloidal pigments prepared essentially as given by Cowdry,[4] with the modifications to be described.

India ink (Higgins Waterproof) was diluted with an equal volume of distilled water just prior to use, or was used undiluted. *Carmine* (Coleman and Bell), 75 gm. in 1000 ml. of distilled water, was shaken for 15 minutes by hand or agitated for 5 minutes in a Waring blendor to form an opaque colloidal solution. *Gelatin* (Bacto-gelatin, Difco), 80 gm. dissolved in 1000 ml. of distilled water at 100° C. and then cooled to 40° C., was added to the carmine solution. *Prussian blue* (Tieman's Soluble Blue, Coleman and Bell), 100 gm. in 1000 ml. of distilled water, was shaken by hand for 10 minutes and then added slowly with stirring to 1000 ml. of gelatin solution as previously prepared. The sediment, after a few days at 37° C., was discarded. The supernatant could then be forced through the finest capillaries. Gelation of the solutions tended to occur at room temperature. Prior to use they were heated to 60° to 80° C. and then cooled to 30° to 40° C. With a few thymol crystals as preservative, both solutions remained stable for months.

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In *living* rabbits with hepatic tumors, India ink was used to trace the flow of portal venous and hepatic arterial blood. Under ether anesthesia a midline abdominal incision was made, exposing the liver and adjacent viscera. India ink in amounts of 2 to 5 ml. was injected into either the hepatic artery or a large tributary of the portal vein. The period of injection was 5 to 10 seconds. Immediately after injection the chest was opened rapidly and the heart incised to prevent circulation of ink.

Living etherized mice were given injections into the portal vein in a similar manner, using 0.1 to 0.2 ml. of ink over a period of 2 to 5 seconds. The hepatic artery was too small to be injected by this method.

Tumor-bearing livers of animals or of human subjects at necropsy were injected by means of an infusion apparatus (Fig. 4) with which it was possible to wash out hepatic artery and portal vein simultaneously with physiologic salt solutions at different pressures, and then switch to the carmine and Prussian blue solutions at these pressures. However, since neither preliminary washing nor maintenance of a physiologic ratio between arterial and venous injection pressure was found to be important, both procedures were soon abandoned. Injections were performed at pressures of 80 to 150 mm. of Hg. When artery and vein were injected simultaneously, the pressure was usually kept the same in both vessels.

The hepatic artery of necropsied mice was too small to be injected directly. The needle was inserted into the aorta just below the celiac artery. Injection was performed with the opening of the needle at the level of the celiac artery. The aorta was ligated above and below this point.

In all specimens injection was continued until the pigments flowed from the hepatic vein. This usually required 5 to 10 minutes. All external vessels were then clamped or ligated, and the entire liver was fixed in 20 per cent formalin for dissection. Livers from human necropsy cases, because of their large size, were placed in ice-cold 20 per cent formalin for several hours in order to gel the injection masses completely. They were then cut into slices 1 to 2 cm. thick and allowed to fix at room temperature.

The degree of staining of the liver surface and of visible tumors was noted throughout the period of injection. After fixation the liver was sliced and the freshly cut surfaces examined under a dissecting microscope. In microscopic sections lightly counterstained with hematoxylin, the injected pigments were seen to be localized within vessels of all calibers.

For the preparation of corrosion specimens, the injection mass was made by dissolving sheet vinylite in a mixture of equal parts of acetone and amyl acetate, to a concentration of 8 per cent. Sudan III or Oil Blue BNA were used to color the mass. After injection the specimen was left in several changes of 95 per cent alcohol in order to extract the amyl acetate. The regions to be studied were then cut out and corroded in concentrated HCl.

Results

Neoplasms Experimentally Induced in the Liver

Tumors were produced by injecting suspensions of tumor cells into the portal vein, hepatic artery, or liver parenchyma of rabbits, and the portal vein or liver parenchyma of mice.

The Vx2 strain[5,6] of carcinoma of rabbits* has been maintained by transplantation into the muscles of the hind leg of rabbits. For the present experiments, actively growing tumor was squeezed through a 40-mesh stainless steel sieve into Ringer-Tyrode solution to form a suspension of single cells and small particles. Through a midline abdominal incision, etherized rabbits were given injections of 0.2 to 0.5 ml. of suspension into either the hepatic artery, portal vein, or liver parenchyma.

A strain of methylcholanthrene-induced spindle cell sarcoma of mice has been maintained by serial subcutaneous inoculation. Suspensions of this tumor, prepared as previously stated, were injected through a 27-gauge needle into either a large tributary of the portal vein or the liver itself, in amounts of 0.05 to 0.15 ml.

Rabbits were sacrificed 3 to 8 weeks after inoculation, mice in 2 to 4 weeks, and their livers injected with the colloidal dyes.

Table I shows the conclusions drawn from the results of the injections. It is evident from the table that the blood supply of the hepatic tumors was not determined by the route through which tumor emboli reached the liver. The supply was arterial, whether emboli were injected into the hepatic artery or the portal vein, and also arterial when tumor was injected directly into the liver parenchyma.

In one half of the animals shown in Table I the colloidal pigments were injected into both hepatic artery and portal vein; the other half received pigment only in the portal vein. In other animals, not shown in the table, the patency of tumor vessels was demonstrated repeatedly by means of arterially injected pigments.

* Obtained from the laboratory of Dr. John G. Kidd, Department of Pathology, Cornell University Medical College, New York City.

The gross appearance of liver injected with pigment through the portal vein, whether during life or after death, is illustrated in Figure 1. The liver takes on the color of the pigment, whereas the tumors remain uncolored. In contrast, pigment injected into the hepatic artery (Fig. 2) colors chiefly the tumors and hepatic tissue adjacent to them. The microscopic appearance of such injected livers is shown in Figures 7, 8, and 9.

The question arose: How is the portal blood flow through tumor-bearing regions of liver eliminated? Microscopic sections showed nu-

TABLE I

Blood Supply of Neoplasms Inoculated into the Livers of Rabbits and Mice by Various Routes

Site of injection of tumor cell suspension	Number of animals		Blood supply of tumors*	
	Rabbits	Mice	Hepatic artery	Portal vein
			%	%
Hepatic artery	4	0	100	0†
Portal vein	6	1	100	0†
Liver parenchyma	1	1	100	0

* The blood supply was determined by the number of vessels per low-power field that were filled by the respective arterial and portal injection masses.

† A small portal blood supply was noted in one rabbit tumor of the several hundred examined.

merous branches of the portal vein, of all sizes, in process of occlusion by invading tumor cells. These changes are illustrated in Figures 10 and 12. Completely occluded portal branches could be distinguished some distance within the tumor. No evidence of occlusion or invasion of arterial branches was seen.

Microscopic examination showed also that many tributaries of the hepatic vein, at the edge of a growing tumor, were invaded or occluded by tumor cells (Fig. 11). The results of retrograde injection of pigment into the hepatic vein indicate that this process of occlusion tends to become complete. In 4 rabbits so injected, although the pigment rapidly traversed the hepatic vessels and appeared in the portal vein, only a few vessels of the tumors were injected. These were small and near the surface of the tumors.

Corrosion preparations made after injecting colored vinylite into the hepatic artery and portal vein indicate that the vasculature of the tumors is very scanty as compared to that of the surrounding liver. In the preparations illustrated in Figures 5 and 6 the tumors appear as "holes" in the rich network of hepatic vessels.

Neoplasms Metastatic to the Liver

Human. Thirteen livers with metastatic carcinomas found at necropsy were injected by the methods which have been described. The blood supply of the tumors was shown to be predominantly or exclusively arterial. The results of injection of the livers of 11 of these subjects are shown in Table II.

TABLE II

Blood Supply of Neoplasms Metastatic to the Liver Found in Eleven Human Subjects at Necropsy

Primary tumor	Approximate number of metastases examined	Size of metastases	Blood supply of tumors*	
			Hepatic artery	Portal vein
		mm.	%	%
Adenocarcinoma, breast	300	10–30	80	20
Undifferentiated carcinoma, breast	50	1–10	95	5
Adenocarcinoma, bronchus	200	2–35	100	0
Adenocarcinoma, bronchus	500	2–50	100	0
Undifferentiated carcinoma, kidney	200	2–35	95	5
Squamous cell carcinoma, cervix	10	20–50	100	0
Adenocarcinoma, sigmoid	10	0.5–1	80	20
Adenocarcinoma, sigmoid	10	1–3	100	0
Adenocarcinoma, stomach	50	2–100	100	0
Adenocarcinoma, rectum	2	15 and 50	95	5
Adenocarcinoma, colon	1	7	100	0

* The blood supply was estimated by determining the number of vessels per low-power field that were filled by the respective arterial and portal injection masses.

Interpretation was sometimes difficult with human necropsy material because of varying degrees of post-mortem autolysis and intravascular clotting. Nevertheless, in only 2 cases was autolysis sufficient to allow some leakage of pigment through capillary endothelium into tissue spaces. Because of scattered blocking of vessels by clots, it was necessary to eliminate from the series all tumors occurring in regions of the liver not reached by both injection masses.

Retrograde injection through the hepatic vein was done in 3 cases, in 2 of which the hepatic artery was also injected and in one the portal vein. All showed only very slight penetration of the injection mass in the hepatic vein into tumor vessels, and these were located near the surface of the tumor. Similar results were obtained with the portal mass. The arterial mass, however, penetrated vessels throughout the tumors.

Microscopically, occlusion of portal branches due to invasion by growing tumor cells was found in some of the metastatic tumors and was similar to that seen in the experimental tumors. In addition, the 3 livers injected through the hepatic vein showed invasion and blockage of tributaries of this vessel by tumor cells.

One *leopard frog* having 5 liver metastases from a primary adenocarcinoma of the kidney[7] was studied. Carmine injected into the portal vein produced intense red staining of all liver sinusoids. The tumors remained colorless except for a few lightly colored vessels in one of the smaller tumors.

Neoplasms Primary in the Liver

Five rats having primary hepatomas and cholangiomas induced by feeding p-dimethyl-amino-azobenzene* were killed with ether and injected immediately with carmine and Prussian blue as has been described. Each liver had two or more white tumor masses, 3 to 25 mm. in diameter, that had the gross and microscopic appearance of malignant growths. Only such tumors are considered in the results shown in Table III.

TABLE III

Blood Supply of Primary Liver Tumors and Surrounding Cirrhotic Liver in Five Rats Fed p-Dimethyl-amino-azobenzene

Type of tissue	Number of animals	Blood supply of tissue*	
		Hepatic artery	Portal vein
		%	%
Liver cell carcinoma (hepatoma)	1	85	15
Liver cell carcinoma (hepatoma)	4	100	0
Bile duct carcinoma	2	100	0
Mixed liver cell and bile duct carcinoma	3	100	0
Cirrhotic (scar) tissue	5	90	10
Nodules of regenerating liver	5	40	60

* The blood supply was estimated by determining the number of vessels per low-power field that were filled by the respective arterial and portal injection masses.

It can be seen from Table III that only liver cell carcinomas retained some portal blood supply. Usually this was small. Bile duct carcinomas and mixed carcinomas were supplied entirely by the hepatic artery.

Rats fed p-dimethyl-amino-azobenzene develop severe cirrhosis with

* Obtained from the laboratories of Dr. Julius White and Dr. Harold Stewart of the National Cancer Institute.

large nodules of regenerating liver cells. These nodules, like regenerating liver, had a predominantly portal supply. In contrast, the abundant, dense, white scar tissue between the regenerating nodules was supplied entirely by the hepatic artery. Many of the scarred regions contained islands of hyperplastic bile ducts. An additional rat fed p-dimethyl-amino-azobenzene had severe cirrhosis but no neoplastic growths. It was injected with carmine through the hepatic vein. All regions of this liver, including scars, stained bright red quickly and uniformly, indicating that scarred areas, in contrast to tumors, have adequate venous drainage.

Miscellaneous Benign or Non-Neoplastic Lesions of the Liver

During the course of the previous experiments, 5 cases having benign or non-neoplastic lesions in the liver were encountered, in addition to the rats showing cirrhotic scarring. Injections were performed soon after death, using the same methods as for malignant lesions.

Three hemangiomas in a human subject had a mixed blood supply. These lesions, grossly and microscopically benign, consisted of numerous dilated blood channels apparently having connections with both hepatic artery and portal vein. On the other hand, scar tissue in the rabbit, mouse, and rat, and foreign body granulomas in the mouse, were supplied chiefly or entirely by the hepatic artery.

Discussion

Results similar to those reported here were obtained by Wright[8] on livers from human necropsy cases showing cirrhosis and metastatic cancers. Wright further concluded that the main venous drainage from the cancers was into the portal rather than the hepatic veins. This interpretation could not be made from the present experiments.

According to Willis,[9] most carcinomas metastasizing to the liver from the alimentary tract reach this organ via the portal vein. However, the results obtained in rabbits suggest that in man emboli reaching the liver via the hepatic artery could become established and that their blood supply would then be the same as in tumors arriving via the portal vein.

Does the arterial supply of tumors indicate that neoplasms stimulate only the proliferation of vessels of an arterial type in the formation of tumor stroma? If this were the case, tumors in the lung should be supplied by the bronchial rather than the pulmonary artery, since the latter carries venous blood. Wright[10] found only bronchial arterial proliferation in tumors of the lung studied at necropsy. Others,[11,12]

also using human material, have concluded that primary (bronchogenic) carcinomas are supplied by the bronchial artery only, whereas metastatic tumors receive blood from the pulmonary artery as well. Our own results[13] with rabbits support the last conclusion. The problem requires further study by refined injection methods.

One additional organ, the amphibian kidney, has a dual blood supply. It would be of interest to determine whether renal carcinoma of the leopard frog,[7] a fairly common neoplasm, is supplied by the renal artery, the renal portal vein, or both.

In the liver the experimental results can be largely accounted for by the well known ability of neoplasms to invade veins. Arteries are rarely invaded.[14] Branches of the portal vein as well as tributaries of the hepatic vein are progressively invaded and obliterated, so that the tumor becomes white and bloodless. This relative avascularity of a number of neoplasms has recently been confirmed by means of radio-iron.[15] Only the arteries tend to persist. Blood pumped into the neoplasm escapes by devious, as yet unobliterated, channels to its periphery.

There are several practical considerations based upon the reported findings: (1) In the absence of knowledge concerning the vasculature of hepatic tumors, one might mistakenly think them to be well situated for chemotherapy by mouth, since blood from the intestine goes directly to the liver. Actually, of course, this blood would by-pass the neoplasms and would reach them only after recirculation. (2) Would ligation of the hepatic artery cause the regression of hepatic neoplasms by cutting off their blood supply? This possibility was tested in a small series of rabbits having Vx2 carcinoma. Regression did not occur. However, liver has a collateral arterial supply derived from diaphragmatic and other vessels, so that the arterial supply to the neoplasms was not completely eliminated.

Summary and Conclusions

By means of injection experiments it was shown that malignant neoplasms growing in the liver tend to acquire an exclusively arterial blood supply.

The neoplasms tested were as follows: Vx2 carcinoma of rabbits, T-241 sarcoma of mice, a variety of metastatic carcinomas found at necropsy in human cases, metastases of spontaneous carcinoma of the kidney of the frog, and primary hepatomas and cholangiomas of rats induced by p-dimethyl-amino-azobenzene.

The blood supply of transplanted rabbit and mouse tumors was

arterial, whether inoculations had been made into the hepatic artery or portal vein or directly into the hepatic parenchyma.

Much if not all of the failure of portal blood to supply tumors growing in the liver is due to progressive invasion and occlusion of portal branches by tumor cells.

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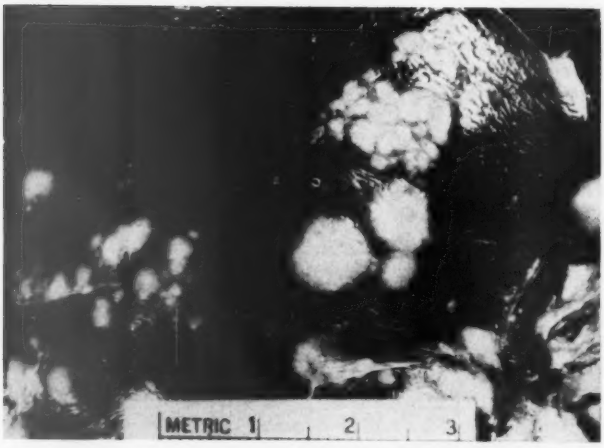
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIG. 1. Surface of the liver of a rabbit 4 weeks after the injection of a suspension of Vx2 carcinoma into the portal vein. Before the animal was sacrificed, India ink was injected into the portal vein under ether anesthesia. The tissue appears black, whereas the tumors remain white.

FIG. 2. Liver of a rabbit injected with Vx2 carcinoma as in the specimen shown in Figure 1. Before sacrificing the animal, India ink was injected into the hepatic artery under ether anesthesia. The tissue shows only scattered flecks of ink, whereas the tumors are stained black.

FIG. 3. Sectioned surface of human liver containing metastases from adenocarcinoma of the bronchus. At necropsy the portal vein was injected with Prussian blue and the hepatic artery with carmine. The patchy gray appearance of the tumors is due to variable amounts of carmine; no blue was seen in the tumors. In contrast, the surrounding tissue was stained an intense blue.



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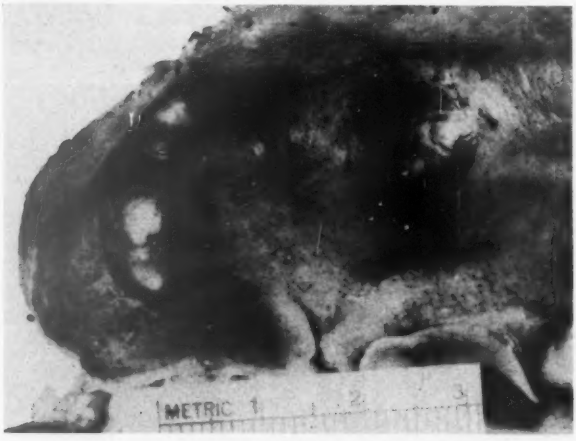

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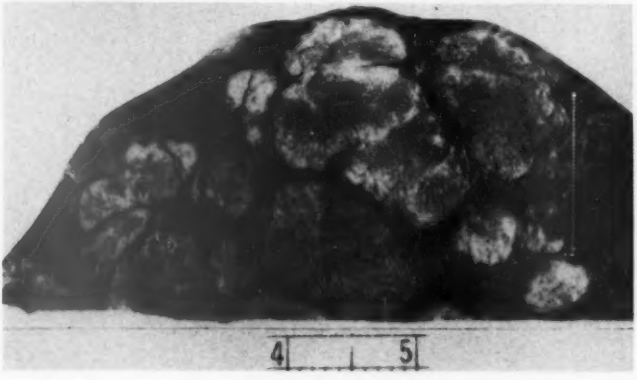

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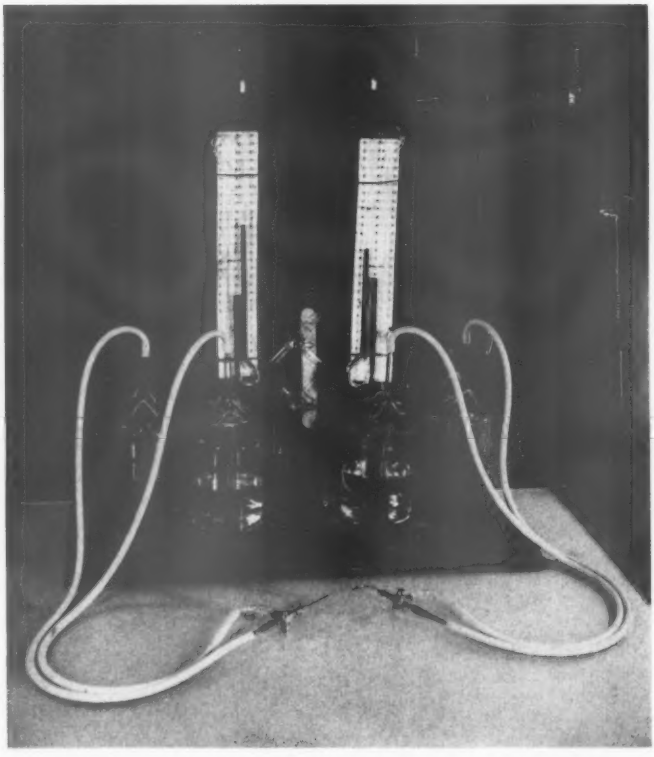
FIG. 4. Apparatus used for injecting artery and vein simultaneously at different pressures.

FIG. 5. Corrosion preparation showing part of the surface of the liver of a rabbit. Near the center of the photograph there is a small Vx2 tumor. Red vinylite was injected into the hepatic artery and blue vinylite into the portal vein. The red vinylite appears white in the photograph and forms a shell consisting of tiny arteries near the periphery of the tumor. The surrounding sinusoids are predominantly blue and appear gray. The gray portal branches that appear to be within the tumor are actually either below it (central part) or are overriding it (upper part). × 4.

FIG. 6. Corrosion preparation of a larger Vx2 tumor in the liver, prepared as was the specimen shown in Figure 4. Here the arteries appear black, the veins white. Near the right hand edge of the fragment there is a tumor containing many arteries, again largely confined to a shell near the periphery. There are no portal branches in the tumor. The tumor is poorly vascularized as compared to the surrounding liver. × 0.5.

FIG. 7. Section of the liver of a rabbit with Vx2 carcinoma. Tumor is shown in the upper left half of the photograph, uninvolved liver in the lower right. Immediately after sacrificing the animal, India ink was injected into the portal vein. The sinusoids contain ink, while there is none in the vessels of the tumor. The sinusoids near the periphery of the tumor are distended with the ink. × 130.



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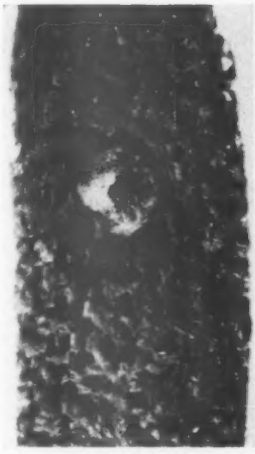

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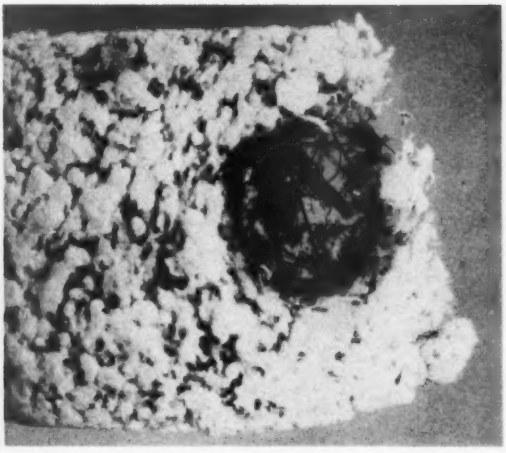

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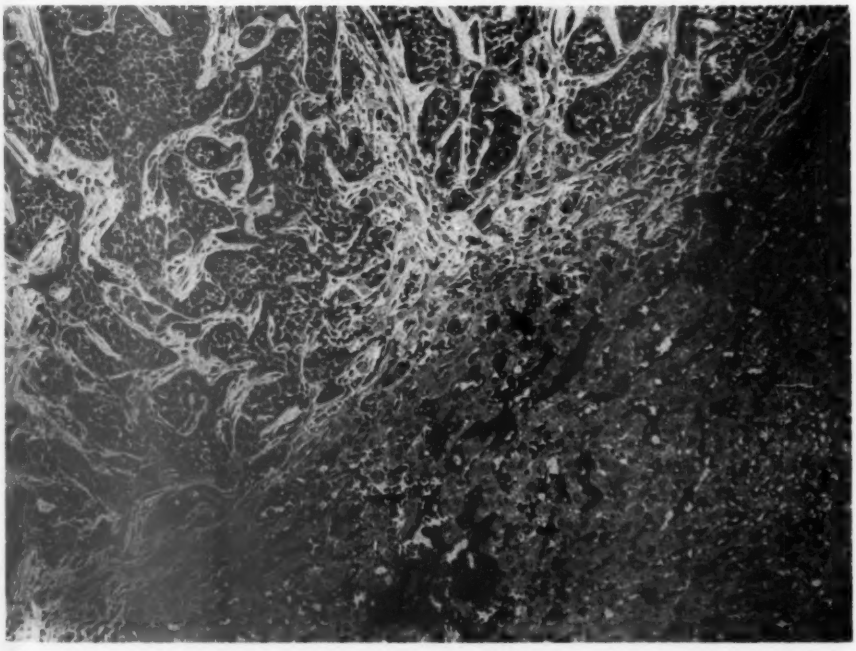

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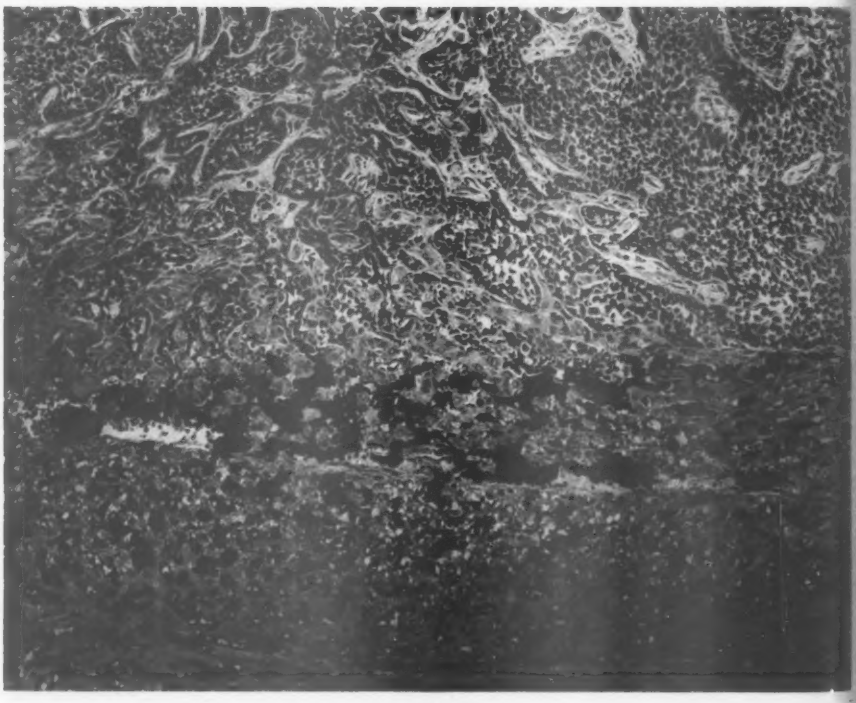
FIG. 8. VX2 carcinoma in liver, prepared as described in the legend for Figure 6. Tumor is shown in the upper half of the photograph, uninvolved liver in the lower half. The sinusoids adjacent to the tumor are dilated with ink. There is none in the tumor. × 130.

FIG. 9. VX2 carcinoma in the liver. After the animal had been sacrificed, India ink was injected into the hepatic artery. The ink fills the sinusoids of the liver (left one third of the photograph) and many vessels in the tumor. × 130.

FIG. 10. Portal space of a rabbit bearing VX2 tumors in other parts of the liver. India ink was injected into the portal vein immediately after death of the animal. The branch of the portal vein is filled with ink, which outlines a mass of tumor cells that have grown into the vessel. × 130.



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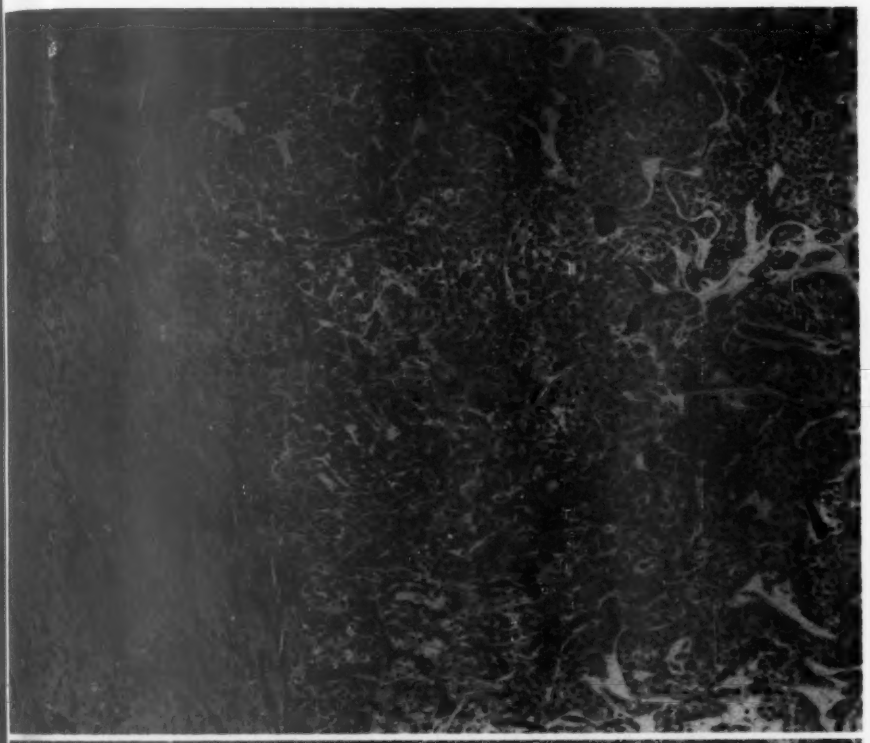

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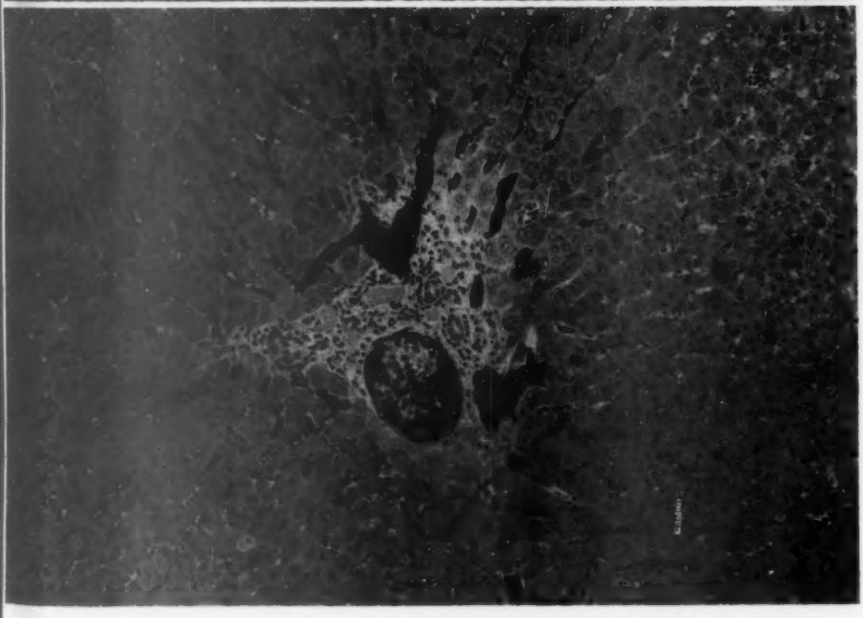

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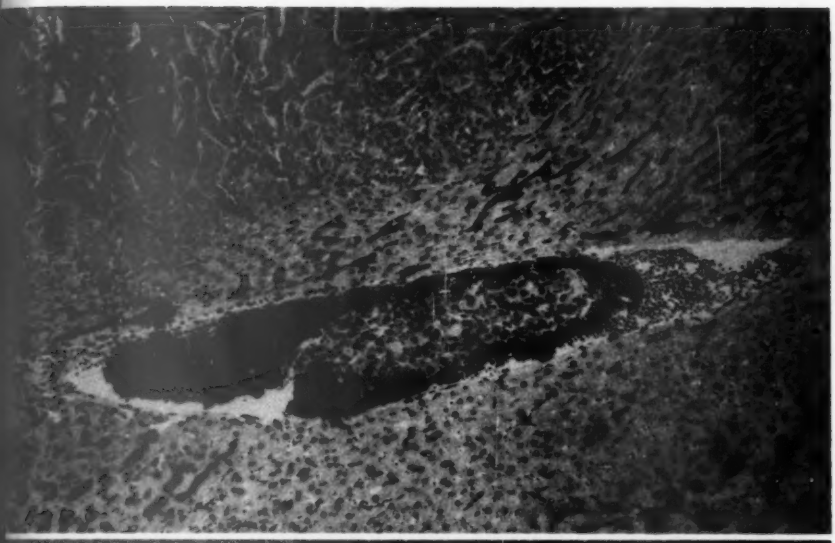
FIG. 11. Liver of a rabbit containing Vx2 carcinoma. Immediately after death of the animal, India ink was injected into the hepatic artery. The ink fills some vessels of the tumor (upper left-hand corner), many sinusoids, and a tributary of the hepatic vein. This vessel (center of photograph) contains a mass of tumor cells completely outlined by ink. × 130.

FIG. 12. Large branch of the portal vein in a rabbit with Vx2 carcinoma in the liver. India ink was injected into the portal vein after death of the animal. The portal branch is almost completely occluded by tumor cells, which apparently have grown in from the tumor on the left. The ink fills the remaining lumen of the portal vessel and many of the sinusoids, but not the vessels in the tumor. × 130.



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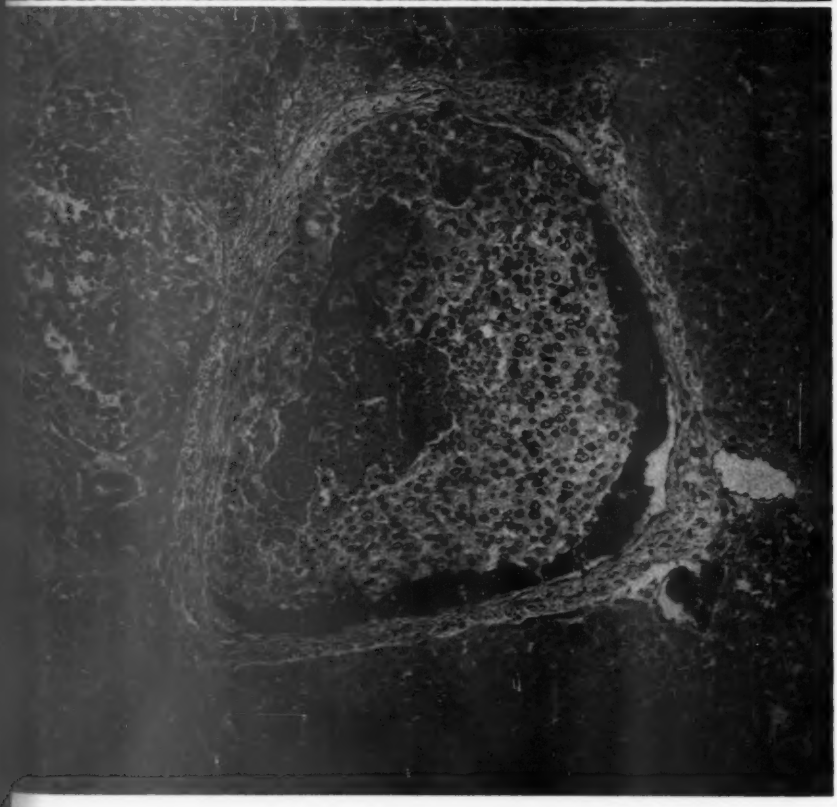

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CYTOPLASMIC LIVER CELL INCLUSIONS FOLLOWING ARTERIALIZATION IN THE DOG *

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During an investigation of the effects of hepatic arterialization (anastomosis of the aorta to the portal vein with an autogenous vein graft) in the dog, distinctive cytoplasmic inclusions were noted within the hepatic parenchymal cells of 5 of 14 arterialized animals. In tissue sections stained with hematoxylin and eosin, they appeared as acidophilic, non-refractile spherules, 2 to 20 μ in diameter, frequently surrounded by an optically clear halo. They occurred singly or in groups without definite geometric pattern in otherwise normal appearing cells. Although they were noted most frequently in cells of the central portion of the liver lobule, their occurrence in peripheral zones was observed also. No similar changes were noted in 5 arterialized and partially hepatectomized dogs, 5 partially hepatectomized dogs with Eck fistula, 5 partially hepatectomized dogs, and numerous controls. The inclusions were unlike those of canine hepatitis, which are principally intranuclear.

The occurrence of cytoplasmic inclusions within liver cells has been referred to by a number of investigators, both in man and in experimental animals. Mallory,[1] in 1901, in his treatise on hepatic necrosis, described filamentous and rod-like bodies within the liver cells which he believed probably represented fibrin. Later, he[2] described the now well known hyaline bodies occurring in the liver cells in alcoholic cirrhosis, which he felt represented a degenerative cellular change. Taniguti[3] described the various inclusions which he observed in human livers and presented a classification of them. He recognized spherical, sharply contoured, homogeneous bodies varying in size from that of a mitochondrial granule to that of a nucleus. Those of a second type were very similar except that they were oval or elongated. Thread-like, slightly bent and often spindle-shaped inclusions, presumably swollen mitochondria, were considered as a distinct group.

In further study of the occurrence of inclusions in liver cells, Pappenheimer and Hawthorne[4] found an incidence of 31.1 per cent in 562 specimens. Included among these were livers of newborn infants, chil-

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dren, and adults. No positive correlation between this cytoplasmic change and associated diseases could be made. It was noted, however, that a lower incidence of inclusions was found in patients suffering from cancer and that none was encountered in the tumor cells of primary liver cell carcinoma. In their experience, the spherical form of inclusion was encountered much more frequently than those of other types. In addition, a survey of various laboratory animals revealed similar inclusions in the livers of the monkey, guinea-pig, and ferret, but not in those of the rabbit, chicken, cat, dog, pig, rat, mouse, or duck. They were unable to arrive at any conclusion as to the nature or significance of these cytoplasmic bodies, although they believed that the filamentous forms were similar to those described by Mallory and probably represented fibrin.

Belt[5] reported the presence of hepatocellular inclusions in patients suffering from burns. Although these patients had received tannic acid treatment, no mention of the possible rôle of this agent or of the nature or significance of these bodies was made.

The majority of experimental studies in which liver cell inclusions have been noted have dealt with liver regeneration and the production of hepatic anoxia. Gurd and Vars[6] observed hepatic necrosis and the presence of eosinophilic inclusions within the liver cells of rats subjected to partial hepatectomy, in which regeneration of liver was retarded by the feeding of a non-protein diet for 14 days prior to the operative procedure. Price and Laird[7] also found inclusions within 24 hours after partial hepatectomy in the rat. They thought that the inclusions were similar to those of Gurd and Vars, but in their experiments the livers were without necrosis. Doniach and Weinbren[8] reported the presence of cellular inclusions following partial hepatectomy which they considered represented protein stores, since none was observed if the animals were starved or given only dextrose in the postoperative period.

Trowell[9] observed vacuolation of the liver cells in a variety of species following the production of hepatic anoxia. This effect was enhanced when the intrasinusoidal pressure was increased or if congestion prevailed. Later, Aterman[10] repeated some of Trowell's experiments as well as performing partial hepatectomy and noticed that following the latter procedure inclusions similar to those reported by Trowell appeared within the regenerating liver fragment. Because of this similarity, he proposed the concept of an existing anoxic state in hepatic regeneration.

Along other lines of investigation it might be noted that Weld, Von

Glahn, and Mitchell[11] observed spherical cytoplasmic inclusions within liver cells following the administration of sheep serum, sensitized horse serum, and streptococcus toxin in rats. Rosin and Doljanski[12] noted inclusions following the administration of allyl formate and urethane, which they believed were erythrocytes from the sinusoids.

The purpose of this report is to present the results of an intensive histologic and histochemical study of the spherical cytoplasmic inclusions occurring in the liver of the dog following arterialization. A comparative analysis between these and similar inclusions, which have been reviewed briefly, will be made and the significance of these bodies will be commented upon.

Material and Methods

Fourteen mature dogs maintained on a stock laboratory diet were subjected to hepatic arterialization. The details of this procedure will be described elsewhere. Essentially it consists of anastomosing the aorta to the portal vein by an autogenous vein graft leaving the arterial supply to the liver intact. These animals were either sacrificed or found dead at various intervals after arterialization (Table I). In

Table I

Time of Observation of Liver Cell Inclusions Following Arterialization

Animal no.	Duration of arterialization	Inclusions, number and size
683	8 hrs.*	Moderate, small
589	1 day*	
675	3 days	
672	7 days	
600	9 days	
695	12 days	
698	20 days	
680	24 days	
634	25 days	Numerous, large
619	25 days	Numerous, moderate
636	30 days	Moderate, small
681	30 days	
618	38 days	Moderate, small
662	42 days*	

* Animal found dead.

addition to arterialization, partial hepatectomy was performed in 5 and the production of an Eck fistula with partial hepatectomy was performed in an equal number. Five more animals were only partially

hepatectomized. These animals were examined after approximately the same intervals as those which were only arterialized.

Portions of liver, pancreas, kidney, and, on occasion, lung, heart, and adrenal gland were fixed in 10 per cent formalin. In addition, in some instances tissues fixed in 95 per cent alcohol and Zenker's acetic fluid were available for study.

The staining procedures were according to the methods in Lillie's *Histopathologic Technic*[13] unless otherwise indicated.

Staining Methods and Results

I. Survey Methods:
- A. Hematoxylin and eosin ..... eosinophilic
- B. Azure eosin ..... eosinophilic
- C. Thionine pH 4, 0.5% in M/100 acetate buffer for 30 seconds ..... not stained
- D. Methyl blue eosin (Mann's stain) ..... blue and bluish purple

II. Collagen Methods:
- A. Masson's trichrome with aniline blue ..... pale blue and red
- B. Heidenhain's azan modification of Mallory ..... blue and red
- C. Mallory's phosphotungstic acid hematoxylin ... blue-black
- D. Wilder's method for reticulum ..... negative

III. Elastic Methods:
- A. Orcein ..... negative
- B. Weigert's stain for elastic fibers ..... negative

IV. Fibrin and Bacterial Methods:
- A. Brown-Brenn method for bacteria[14] ..... negative
- B. Gram-Weigert ..... blue and red
- C. Ziehl-Neelsen ..... pale to moderate red (acid fast)

V. Amyloid Methods:
- A. Crystal violet ..... negative
- B. Congo red ..... negative

VI. Mucin Methods:
- A. Muchematein ..... negative
- B. Mucicarmine ..... negative
- C. Rinehart–Abul-Haj[15] method for acid mucopolysaccharides ..... negative
- D. Periodic acid-Schiff method ..... see IX

VII. Lipids and Unsaturated Compounds:
- A. Oil red O on frozen and paraffin sections at 25° C. and 60° C. ..... negative
- B. Sudan black B on frozen and paraffin sections at 25° C. and 60° C. ..... negative
- C. Baker's acid hematein method for phospholipids. negative
- D. Feulgen-Voigt plasmal reaction on frozen sections for acetal phosphatides ..... negative
- E. Peracetic acid-Schiff reaction for unsaturated compounds ..... red-purple
  1. Pyridine extraction for 24 hours at 60° C. followed by peracetic acid-Schiff ..... no effect
  2. Chloroform methanol extraction for 24 hours at 60° C. followed by above ..... no effect

3. Bromination with bromine in CCl_4 for 2 hours followed by above ..... no effect
4. Bromination with bromine in CCl_4 for 5 hours followed by above ..... moderate reduction
5. Bromination with bromine in CCl_4 for 16 hours followed by above ..... block
   a. CCl_4 control ..... no effect

F. $KMnO_4$-Schiff reaction for ethylenic linkages as well as 1,2 glycols or hydroxylamines ..... red-purple
1. Bromination prior to $KMnO_4$-Schiff ..... no effect
2. Acetylation prior to $KMnO_4$-Schiff ..... moderate block
3. Acetylation and bromination prior to $KMnO_4$-Schiff ..... complete block

G. Lillie-Weil-Weigert myelin stain ..... negative
H. Fischler's method for fatty acids ..... negative

VIII. Protein Methods:
A. Millon's reaction for tyrosine-containing protein ..... red-brown (positive)
B. Ninhydrin-Schiff, a proposed test for demonstration of protein[16] ..... red-purple
C. Diazosafranin method of Lillie, Burtner, and Henson,[17] a coupling reaction of phenols ..... red
1. 2:4 Dinitrofluorobenzene followed by above[18,19] ..... block
2. Performic acid followed by above[18,19] ..... no effect
3. Benzoylation (benzoyl chloride 2:pyridine 38, at 60° C. for 4 hours) followed by above[18,19] ..... block

D. Chromotrope 2R aniline blue method of Roque[20] ..... blue or red; periphery occasionally blue
E. Deamination with HNO_2 (6 gm. $NaNO_2$, 5 cc. glacial acetic acid, water qs. ad 40 cc.) for 24 hours at 25° C. followed by hematoxylin and eosin and chromotrope 2R aniline blue ..... complete block
F. Feulgen nucleal ..... negative
G. Ribonuclease digestion (crystalline ribonuclease 1:10000 Armour Lot #70595X in pH 6 phosphate buffer) for 1 hour at 37° C. followed by Gram-Weigert ..... no effect
H. Pepsin digestion (0.1% control #401003 Difco Lab.) dissolved in N/10 HCl. Sections incubated at 37° C. for 2, 4, 6, 16 hours followed by hematoxylin and eosin and periodic acid-Schiff (*infra vide*) ..... removed in 6 hours
I. Digestion with trypsin (Lot #521569 Fisher Lab.) in M/100 phosphate buffer pH 7.6 for 2, 6, 24, 30 hours ..... no effect

IX. Periodic Acid-Schiff Methods:
A. Hotchkiss[21] method ..... faint pink
B. Periodic acid-Schiff, Lillie variant ..... red-purple
1. Acetylation (acetic anhydride 16:pyridine 24 for 24 hours at 25° C.) prior to PAS ... complete block

2. Bromination (Br_2 1:CCl_4 39 for 2 hours at 25° C.) for 5 hours prior to PAS ..... no effect
3. Digestion with barley malt diastase 0.1% in saline phosphate buffer of pH 6, 1 hour at 37° C. followed by periodic acid-Schiff reaction for demonstration of glycogen ... no effect
4. Digestion with pectinase (Nutritional Biochemicals Corp. Lot #3783) 0.4% in acetate buffer pH 3.8 for 48 and 72 hours at 37° C. followed by PAS ..... negative (no effect)
5. Hyaluronidase (Wyeth Lot #173A, 1500 TRU/mg.) 150 TRU/cc. in pH 5.5 sodium acetate acid buffer for 1 hour at 37° C. followed by PAS ..... no effect
6. Direct Schiff for 10 minutes and 48, 72 hours ..... faint pink after 48 hours

X. Other Methods:
- A. Ferric ferricyanide reduction test for sulfhydryls, phenols, etc.[22] ..... negative
- B. Gomori's methenamine silver method, Burtner-Lillie variant, demonstrating di- or polyhydroxy or NH_2OH aryl compounds .... negative
- C. Winkler-Schultz method for peroxidases ..... negative
- D. Peracetic azure A reaction for cleavage of disulfide bonds[23] ..... negative
- E. Inorganic phosphate method of Bunting[24] (modified Feigl and Serra, and Queiroz-Lopes) ..... negative
- F. von Kossa's for phosphate or carbonate deposits ..... negative
- G. Laidlaw's inclusion method[4] ..... red

XI. Birefringence ..... none

Discussion

From the results of the protein reactions obtained, it is apparent that the cytoplasmic inclusions contain a protein moiety. The positive Millon reaction and the failure of performic acid to inhibit the diazosafranin coupling in the light of effective dinitrofluorobenzene and benzoylation blockade, according to Danielli,[18] indicate tyrosine-containing protein. The affinity for acid dyes demonstrated by the inclusions likewise adds supporting evidence of their protein nature. That the protein is not nucleoprotein is shown by the negative Feulgen nucleal reaction and ineffective digestion with ribonuclease. The peptic digestion observed also suggests a protein structure. The inability of trypsin to affect the staining reactions may be due to the difference in the protein side chain requisites of the two enzymes.[25]

The protein present, however, is in conjugated form, although not as lipoprotein. This is indicated by the failure of the inclusions to stain with oil red O or Sudan black B at 25° C. and 60° C. in both frozen

and paraffin sections. In addition, extraction procedures with hot pyridine as well as hot chloroform-methanol have been ineffective in altering subsequent staining procedures. Myelin stains and the acid hematein method of Baker for phospholipid have also been negative.

The positive peracetic acid-Schiff reaction indicates ethylenic linkages. In addition, the periodic acid-Schiff and permanganate Schiff reactions are strongly positive. This situation and the effects of various blockades upon these reactions (Table II) are analogous to that ob-

TABLE II

Schiff Reactions Obtained after Oxidation with Permanganate, Peracetic, and Periodic Acids

	Direct	Acetylation	Bromination	Acetylation-bromination
Permanganate	+	±	+	−
Peracetic	+	+	Slow block	−
Periodic	+	−	+	−

served in the histochemical studies on ceroid pigment.[26] Another similarity to this material is displayed in acid fastness. However, ceroid is sudanophilic and probably represents highly polymerized unsaturated lipid. The periodic acid-Schiff reaction is shown to be due to the presence of vicinal OH or OH-NH_2 groups in these hepatic inclusions by the effective blockade produced by the acetic anhydride-pyridine mixture (acetylation). The possibility that epoxidation of the ethylenic linkages of the peracetic acid-Schiff reactive material within the inclusions has occurred could well explain the positive periodic acid-Schiff reaction, since epoxides hydrolyze in an acid medium to 1,2 glycols. The presence of epoxidation might account, in part, for the necessity of prolonged bromination for the inhibition of the peracetic acid-Schiff reaction since this structural arrangement limits the number of bonds for bromine addition. Although the periodic acid-Schiff reaction could be due to neutral or acid mucopolysaccharide or glycogen, there is little support offered for this contention by the available data. It has been stated[27] that if positive periodic acid and peracetic acid-Schiff material is found, unless otherwise proved, the presence of epoxide forms of unsaturated compounds rather than glycoprotein or mucopolysaccharide structures must be assumed. Pectinase digestion does not affect the periodic acid-Schiff reaction of these inclusions and glycogen may be excluded by the ineffectiveness of diastase digestion. Other methods for acid mucopolysaccharides are negative.

It may be concluded, therefore, that histochemically the cytoplasmic

liver inclusions may be characterized as being an unsaturated, probably non-lipid, protein complex. This contention can be more convincingly rationalized than ascribing lipoprotein or glycoprotein structure to this material.

The identity of the unsaturated portion is at present not apparent. However, in considering possible naturally occurring, unsaturated, non-lipid substances we were confronted with the unsaturated hydrocarbon squalene ($C_{30}H_{50}$). This unsaponifiable oil was recovered from shark liver oil in 1916 by Tsujimoto.[28] The exact significance of this finding was not apparent at that time although it was speculated that squalene played a rôle in some physiologic process within the liver. Later work by Channon and Marrian[29] led to the recovery of a squalene-like unsaturated hydrocarbon from the livers of man, ox, sheep, horse, and pig. They believed that the material represented a higher homologue than squalene. In addition, Channon[30] noted that when squalene was administered to the rat, the liver cholesterol increased almost 100 per cent. Yet from further studies[31] he was of the opinion that squalene or its higher homologues were not cholesterol precursors. This has been contested by others.[32,33] It has been demonstrated recently that this material, when administered to the rabbit, is in part stored within the liver and in part eliminated by the intestine and kidneys. Some is oxidized and decomposed into succinic and laevulinic acids.[34]

From the early work of Tsujimoto,[28] it was noted that squalene was soluble in ether, carbon tetrachloride, and acetone, and sparingly so in cold alcohol and glacial acetic acid. Yet lipid solvents fail to dissolve the liver inclusions found following arterialization. Several possible explanations for the insolubility of this material, assuming it to be of squalene-like structure, may be offered. First, the material may be a higher homologue of squalene which is less soluble than squalene *per se*. Secondly, the unsaturated material may be linked with an insoluble portion, presumably protein in this case. Lastly, there may be cross-linking within the unsaturated moiety itself with resultant alteration in solubility.

Positive proof of a squalene-like nature for the unsaturated portion of this material is lacking. The possibility is quite attractive but proof lies in further chemical studies as to this substance and its rôle in various states in which inclusions have been noted.

A tabulation of the results of the various staining reactions obtained on the spherical cytoplasmic inclusions as reported by various investigators is presented in Table III. Unfortunately, in some instances these reports are fragmentary. Yet there does appear, in general, to be agreement as to their tinctorial qualities. Whether this represents

TABLE III

Tabulation of Staining Reactions of Spherical Inclusions

	Pappenheimer and Hawthorne[4]	Gurd and Vars[6]	Aterman[10]	Trowell[9]	Doniach and Weinbren[8]	Price and Laird[7]	Weld *et al.*[11]	Fisher and Fisher
Methods	Human, ferret, monkey, guinea-pig	Partial hepatectomy (rat)	Partial hepatectomy; injected CCl_4; ligation of bile duct (rat)	Hepatic anoxia (rat, rabbit, monkey, guinea-pig)	Partial hepatectomy (rat)	Partial hepatectomy (rat)	Streptococcus toxin, sheep serum, anaphylaxis, horse serum, egg albumin (rat)	Arterialization (dog)
Hematoxylin and eosin	Eosinophilic	Eosinophilic		Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic	Eosinophilic
Azan carmine	Orange						Blue and orange	Blue and orange
Phosphotungstic acid hematoxylin	Blue–black						Yellow and blue	Blue–black
Giemsa	Rose–pink						Red	Rose–red
Gram	Positive				Positive	Negative		Many +, few −
Methyl blue eosin	Red to blue–purple						Center red, periphery blue	Blue–purple
Masson	Center red, periphery blue							Blue and red
Mucicarmine	Negative							Negative
Laidlaw	Crimson							Red
Millon					Positive			Positive
Feulgen						Negative		Negative
Hotchkiss			Pink					Pink
Glycogen			Negative	Negative	Negative		Negative	Negative
Sudanophil	Negative		Negative	Negative	Negative except faint Sudan black B		Negative	Negative

a basic similarity of chemical structure cannot be stated with assurance since further exploration might have revealed outstanding differences. Of significance is the lack of glycogen and sudanophilic material in all instances except for the faint Sudan black B reaction obtained by Doniach and Weinbren.[8] This observation was not pursued further and the lipid nature of the inclusions which they described cannot be assumed. They themselves have not claimed a lipid component to be present.

Of some value in addition to the sparse data of histochemical significance are morphologic comparisons of these various inclusions. The spherical bodies reported by Pappenheimer and Hawthorne[4] are smaller, only 2 to 4 μ, than those reported by other investigators. The "watery vacuolation" of Trowell[9] also appears to be morphologically distinct. These "inclusions" are described as for the most part optically clear spaces within the liver cell cytoplasm, frequently the size of a nucleus, but only rarely possessing a faint-staining, plasma-like material within the vacuoles. Whether these two forms represent distinct entities or are variations in the development of the larger, more solid appearing inclusions cannot be proved, although from their distinctive morphologic features the former concept is perhaps more tenable. On the other hand, inclusions noted following partial hepatectomy demonstrate closely similar morphologic and tinctorial qualities in so far as these latter have been investigated. This might be expected as being more than fortuitious since fundamentally similar experimental conditions prevailed. Included within this group are the inclusions we have observed following arterialization of dogs' livers.

Even though it is not within the scope of this paper to elucidate the chemical and morphologic features and significance of the alcoholic hyaline bodies described by Mallory,[2] nevertheless a brief note may be made in this regard since they need to be distinguished clearly from the inclusions studied in this report. Morphologically, they are unlike these. They are characterized as an irregular coarse meshwork within the cytoplasm and without peripheral vacuolation. An outstanding staining difference is their failure to be colorized by the periodic acid-Schiff reaction.

Disagreement, however, has been noted as to the proposed significance of the inclusions observed following partial hepatectomy. Doniach and Weinbren[8] believed that they represent protein stores and that they were different from those reported by Aterman.[10] The latter was of the opinion that the inclusions resembled those described by Trowell[9] during hepatic anoxia. This led to the concept of the existence of a state of anoxia in the regenerating liver fragment following partial

hepatectomy. Aterman also was of the belief that the inclusions were either of glycoprotein or lipoprotein structure, although proof of this was not presented. Price and Laird[7] were of the opinion that the inclusions they noted were similar to those reported by Gurd and Vars,[6] but no reference to their possible significance was made. It seems reasonable to assume that these differences in the interpretation of the significance of these inclusions are less important than the similarities of morphology and staining reactions, albeit incomplete.

The occurrence of what we believe are comparable cytoplasmic bodies in arterialized livers offers another example of a physiologic state capable of producing this change within the liver. Whether this material is an increased, and thus more easily recognizable, naturally occurring component of the hepatic cell, or exogenous material shunted to the liver in large quantity for storage is difficult to say. Further work on the physiologic and chemical alterations occurring in the arterialized state is in progress and will be the basis of a subsequent report.

The inconstant occurrence of these inclusions following arterialization (Table I) is similar to the results obtained with partial hepatectomy as reported by others. Although in the rat following partial hepatectomy, inclusions were found within 48, and most frequently 24 hours, after the procedure, their appearance in the arterialized liver was most frequent at 25 to 26 days. However, a moderate number of inclusions were noted on one occasion 8 hours following the procedure. As in the case of other reported inclusions, we could find no relationship between this cytoplasmic change and morbidity or mortality. Sections of other tissues studied were without change.

Summary

Spherical cytoplasmic liver cell inclusions similar to those observed by others following partial hepatectomy in other species were noted in one third of the dogs subjected to arterialization.

Histochemical study permits them to be characterized as an unsaturated, probably non-lipid, compound protein complex. The possibility of this material being similar to the unsaturated hydrocarbon squalene is discussed.

A comparison of these hepatic inclusions with those reported as occurring under other conditions is made.

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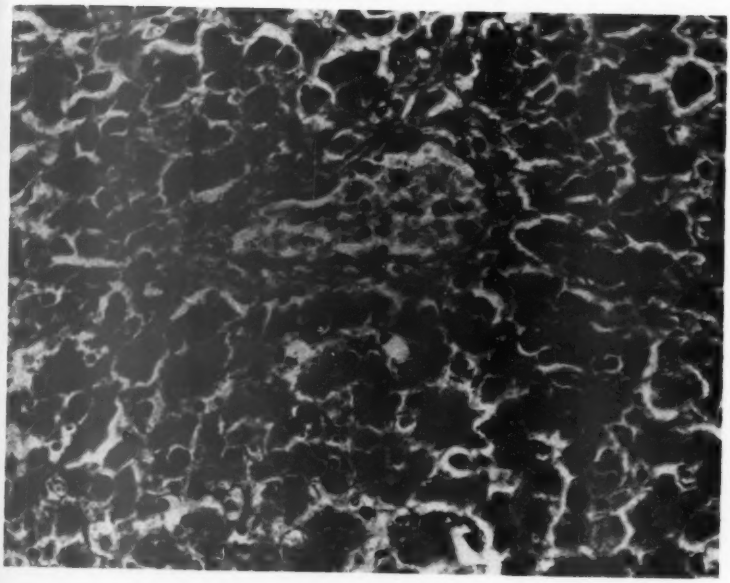
[*Illustrations follow*]

LEGENDS FOR FIGURES

FIGS. 1 and 2. Sections of liver stained by the periodic acid-Schiff method demonstrating cytoplasmic inclusions in liver cells following hepatic arterialization in the dog. × 320 and 670.



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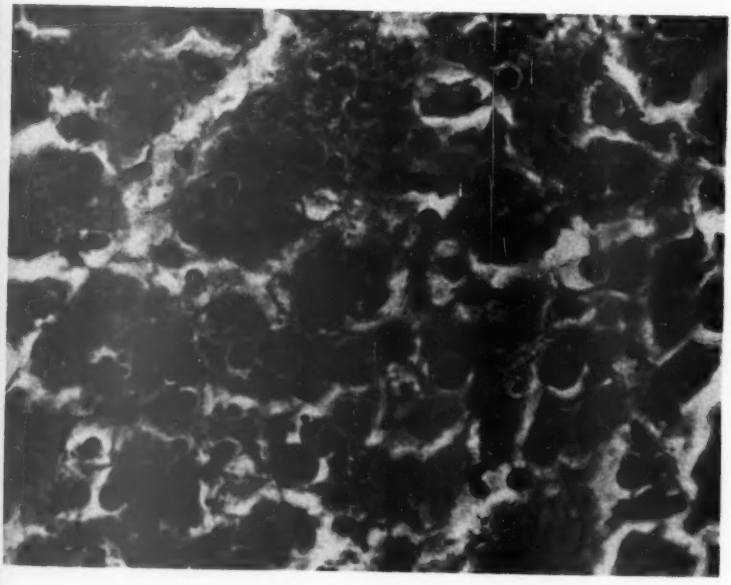

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THE DETERMINATION OF THE TOTAL WEIGHT OF SILICA, AND ITS CORRELATION WITH TISSUE REACTION, IN THE LUNGS OF EXPERIMENTAL ANIMALS *

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It has long been recognized that virtually no correlation is demonstrable between the concentration of silica in pulmonary tissue, reported as a percentage of dry tissue or of ash, and the degree of tissue reaction to silica observed in sections of the same pulmonary tissue. Indeed, it has been noted[1] in some instances that lungs revealing no evidence of reaction to silica contained a larger percentage of silica than did certain other lungs which showed a characteristic silicotic reaction. Such observations have led some investigators[2] to suggest that there may be wide individual variation in tissue susceptibility to the injurious action of silica.

In order to investigate this concept, it became desirable to determine the total weight of silica in the entire lung rather than merely the concentration (per cent) of silica in the dry tissue or in the ash. The total weight of silica could be determined by analyzing the entire lung, but in that procedure there would be no opportunity to prepare histologic sections for determining the degree of tissue reaction. In order to correlate analytical results and histologic findings it was necessary to devise a technique by which data for the total weight of silica could be compared with the histologic observation. A simple procedure has been developed by which this comparison can be made. The technique involves the analysis of a sample which is a known proportion of the entire lung. The amount of silica found in the sample is then multiplied by the ratio of the weight of the entire lung to the weight of the analyzed tissue and the product represents the silica content of the whole lung. The method is, of course, applicable for the determination, in absolute quantity, of any constituent of an organ.

METHOD

In the handling of the lungs of experimental animals in carrying out this procedure, considerable care must be exercised. The steps are listed in chronologic order:

1. The lungs are fixed in the usual manner by injection of 10% formalin through the trachea; the injected lungs are then immersed in 10% formalin.

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2. After fixation, routine blocks for histologic examination are cut. In this laboratory the usual procedure involves the preparation of a single block in a frontal plane, including both lungs and the bronchi, trachea, and tracheobronchial lymph nodes.

3. The lymph nodes, fat, and major bronchi are dissected from the portion of tissue to be analyzed and are discarded.

4. The entire lungs, including the histologic blocks, but excluding the discarded lymph nodes, fat, and major bronchi, are weighed and the weight is recorded as A.

5. The portion of tissue to be used for chemical analysis is weighed and the weight is recorded as B.

6. Portion B is dried overnight at 105° C. and then ashed in a muffle furnace at 450 to 500° C. The total silica content of the ash is determined by standard methods, employing fusion with sodium carbonate and two evaporations with hydrochloric acid.

7. After analysis of portion B, the weight of silica in the entire lung is calculated by multiplying the silica found in the analyzed portion by the ratio A/B. Similarly, the weight of ash of the entire lung can be calculated by multiplying the ash of the analyzed portion by the ratio A/B.

Unnecessary handling of the tissue between step 2 and steps 4 and 5 must be avoided because excessive loss of fluid from the tissue to be used for histologic study, as compared with the portion to be used for chemical analysis, would introduce error. In practice, the tissue is handled and examined as little as possible before it is weighed and each piece of tissue is placed on a piece of absorbent paper in order to remove surface fluid before being weighed. After the weighing has been accomplished, the tissue can be examined freely in the gross, provided none of that intended for analysis is discarded.

TABLE I

Comparison of Calculated and Determined Silica Content, by Weight

	Guinea-pig			
	A	B	C	D
	mg.	mg.	mg.	mg.
SiO_2, chemical determination	41.2	46.2	46.0	34.4
SiO_2, calculated	46.0	52.0	51.5	40.8

RESULTS

The technique just described has been checked in a small number of animals (four) in which the histologic blocks were analyzed chemically for silica content instead of being used for sectioning. In these animals it is thus possible to compare the data for calculated silica content with the actually determined total content, obtained by adding the weight of silica found in the histologic block to the weight found in those portions routinely used for analysis. These results are presented in Table I.

It will be seen in Table I that the calculated total weight is higher than the determined one. It is important to note, however, that the excess weight by calculation is rather constant at about 6 mg., or approximately 12 per cent of the total. This excess is readily explainable in view of the technique used. The explanation is presented in a footnote* in order not to obscure the point which it is desired to stress, namely, that the calculated figure is reasonably accurate and the difference between it and the determined one is consistent, so that data obtained by the technique described can be compared and analyzed with confidence in their validity.

This technique is being applied in a number of experiments which will be described in subsequent reports. The results in one recent experiment are presented here (Text-fig. 1) in order to illustrate the potentialities of the technique. In Text-figure 1 are shown the values, obtained in the experiment, for silica as per cent of ash in comparison with the values for total weight of silica and total weight of ash. Photographs of tissue sections (Figs. 1, 2, 3, and 4) make it possible to evaluate the correlation in individual animals between the total weight of silica and the tissue reaction.

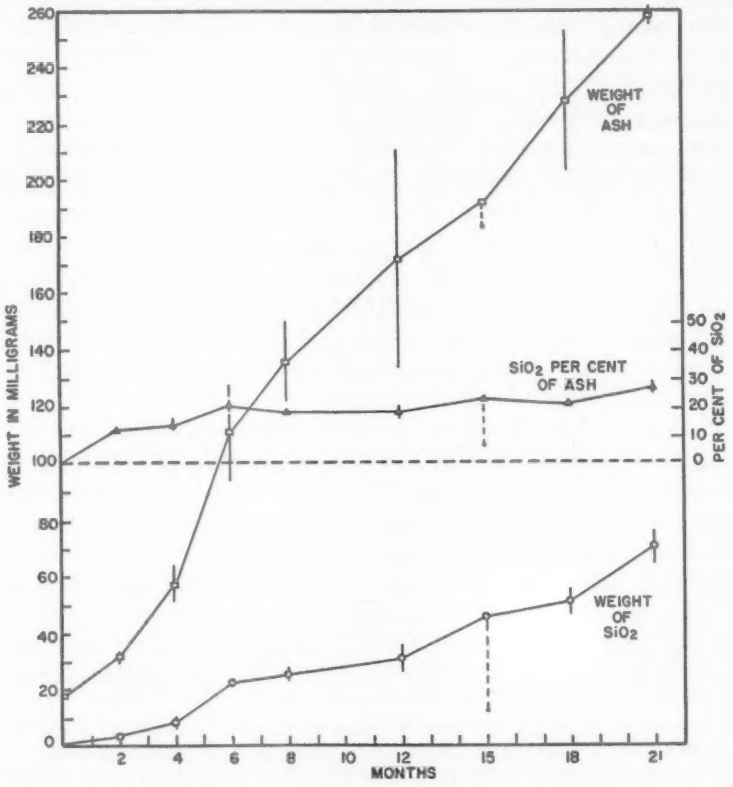
The nature of the tissue reaction in the present experiment need not be reported in detail, since the purpose of this report is limited to the presentation of a new technique. The dust consisted of crystalline free silica and was capable of inciting a fibrous tissue reaction. The animals lived in a dust room throughout the 21 months' duration of the experiment and a dust cloud was maintained in the atmosphere of the room for 8 hours daily, 5½ days each week. The dust concentration was determined regularly, both on the basis of particle count and on the basis of weight per cubic foot of air. The over-all average for

* The consistent excess of the calculated silica content over the determined content results from the fact that the routine histologic block is weighed with the tissue for analysis in step 4. Since it is desirable for that block to include the mediastinal tissue, such as trachea, bronchi, and bronchial lymph nodes, these structures are not dissected away and their weight exaggerates the figure for total lung weight by about 1 to 2 gm. Therefore, in the ratio A/B, A is slightly excessive and the product of the weight of silica found in portion B times A/B is too large.

As a matter of fact, in the special test presented in Table I the mediastinal tissues were dissected from the histologic block and weighed separately. In calculating the data presented in Table I, the weight of the mediastinal component of the histologic block was included in the total lung weight. If the total weight of the lung is corrected by subtracting the mediastinal component, the calculated total silica for each animal is within 2 mg. of the determined value. Again, calculated values are consistently higher than those determined. This slight excess is of no practical importance, but deserves academic consideration. It is believed to be an indication of the fact that silica is not evenly distributed through the lung, but tends to localize excessively in ventral areas.

the dust count was 167 million particles per cubic foot of air and the average concentration on a weight basis was 1.6 mg. per cubic foot of air.

Text-figure 1 is self-explanatory. It will be noted that the average values for silica reported as per cent of ash rise rapidly in the early



Text-fig. 1. Ash and silica content of lungs of guinea-pigs exposed to silica dust. The sloping lines on the chart pass through points which represent the average of the values obtained for two animals at each time interval. The vertical lines above and below each point indicate the range of the values. The baseline for plotting the percentage values is different from that for weight. The weight units are on the left, and the percentage units on the right. In one of the animals killed at 15 months, the data obtained were obviously erroneous, and were not averaged. The vertical broken lines indicate the values obtained in this animal.

months of the experiment, reaching 20.9 per cent in 6 months, and then level off and remain essentially constant in the later months. In contrast, there is a gradual but continuous rise in the total weight of silica

in the lungs, which reaches 70.3 mg. at 21 months. The reason for the leveling off of the percentage data is revealed by the line representing the total weight of lung ash, which also rises continuously but at an increased rate as compared with the total weight of silica. Thus, although the weight of silica increases, the weight of ash tends to increase faster and the percentage (weight of silica divided by weight of ash) remains constant.

Comment

The use of the wet weight of tissue in the calculations seems at first quite unreliable because of the unknown amount of extraneous material present. However, since the data for weights of tissue occur in both the numerator and the denominator of the ratio used in the calculations, this extraneous material is of no moment. The following equations illustrate this point:

When a lung having weight W is divided into two parts, M and N, the following equation expresses the condition:

$$M+N=W \qquad (I)$$

If a quantity X, for example the fixing fluid, is added to W, and the result is divided into two parts, the equation is:

$$M+\frac{M}{W}X+N+\frac{N}{W}X=W+X \qquad (II)$$

The following equation can be used to test whether the presence of X exerts any effect on the proportion of W to M:

$$\frac{W+X}{M+\frac{M}{W}X}=\frac{W}{M} \qquad (III)$$

Algebraic manipulation of equation (III) shows that X does not alter the proportion of W to M, since cross multiplication of the numerators and denominators results in identical expressions on the two sides.

It should be noted that in carrying out the procedure it is necessary to assume that the silica deposited in the lung is evenly distributed. While it has not been fully demonstrated that this is the case, it may be mentioned that the same assumption is made in calculations used for determining the silica concentration as percentage of lung ash or dry tissue.

It is important to emphasize again the need of care in the handling of pulmonary tissue before weighing the portions. Even with all possible care, complete accuracy in the weighing of wet animal lungs is not possible. However, the good correlation between the calculated and the determined silica content of the entire lung, and between the

silica and the degree of tissue reaction (Figs. 1, 2, 3, and 4), reveals the superiority of the method in comparison with the usual one in which the silica is reported as a percentage of the tissue or of the ash.

Examination of Text-figure 1 reveals that the reason for the failure of correlation of silica concentration data with tissue reaction is the extreme variation in lung ash, which introduces a very great error into the calculation. It can be shown readily that this is also the case for dry tissue data. Thus, it is clear that as tissue reaction to silica develops, the inorganic components of that reaction in the lung ash dilute the silica so that, even though it is increasing in amount, its percentage concentration remains essentially constant. Another aspect of the relationship between ash weight and silica weight suggests itself: it seems possible that if the total weight of silica in the lung is subtracted from the total ash of the lung, the remainder may represent an objective measure of the degree of tissue reaction present. At least this would be the case in the absence of histologic evidence of infection, tumor, or other causes of tissue reaction.

These results suggest that there is probably little individual variation among experimental animals of a given species in the susceptibility of tissue to toxic effects of inhaled quartz dust. Instead, it appears that there is a substantial individual variation in the rate of accumulation of the inhaled dust, even in animals exposed to the same dust under identical conditions.

The availability of data for the total weight of silica, or of other inorganic substances in the lung, makes possible many studies concerning the accumulation of dust in, and the elimination of dust from, pulmonary tissue. The method for determining the total amount of silica in the lung and correlating it with the histologic appearance is being used for such studies, which will be the subject of future reports. It should be added that the same principle is being applied in the study of pneumoconiosis in necropsy material. Slight modifications of the technique are necessary, however, because of the larger size of human lungs.

Summary

A method is described by which the weight of silica, or other components, in pulmonary tissue can be calculated and correlated with microscopic pathology.

An animal experiment is reported to demonstrate the correlation between silica content and tissue reaction. The correlation between total silica content on a weight basis and tissue reaction is shown to be far

better than the correlation between silica content on a percentage basis and tissue reaction.

Reasons for this long recognized failure of silica content, reported as a percentage, to correlate with tissue reaction, are pointed out.

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[*Illustrations follow*]

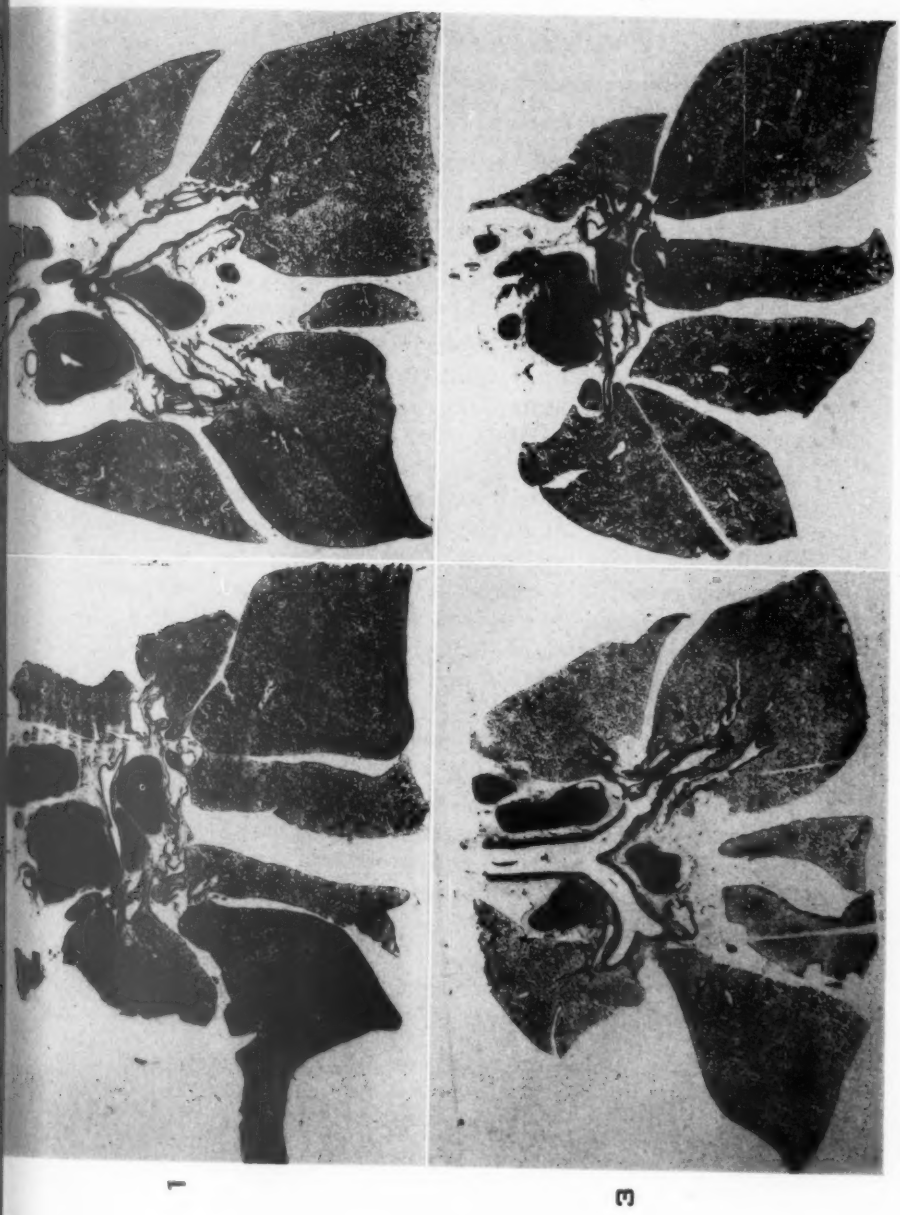
LEGENDS FOR FIGURES

FIG. 1. Frontal plane section through both lungs of a guinea-pig killed after 12 months of exposure to silica dust. × 2. Silica content by weight, 35.9 mg.; by percentage of ash, 16.9.

FIG. 2. Frontal plane section through both lungs of a guinea-pig killed after 12 months of exposure to silica dust. × 2. Silica content by weight, 25.9 mg.; by percentage of ash, 18.7. Comparison of Figures 1 and 2 reveals that the tissue reaction is distinctly more intense and more extensive in the lungs of the animal having the greater silica content by weight, Figure 1.

FIG. 3. Frontal plane section through both lungs of a guinea-pig killed after 6 months of exposure to silica dust. × 2. Silica content by weight, 22.2 mg.; by percentage of ash. 23.5.

FIG. 4. Frontal plane section through both lungs of a guinea-pig killed after 18 months of exposure to silica dust. × 2. Silica content by weight, 45.8 mg.; by percentage of ash, 22.5. Comparison of Figures 3 and 4 reveals that although the percentage data are about the same at 6 months as at 18 months, the tissue reaction is far more pronounced in the latter animal, the lungs of which contained about twice as much silica as did those of the former animal. The reaction shown in Figure 4 is more pronounced than that in Figure 1. (The lower lobe on the left in Figure 1 failed to expand when fixing fluid was injected.)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